HIGHER ORDER CHROMATIN STRUCTURE
OF THE TYROSINASE GENE LOCUS

by

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Date Dec 73, 1998
Abstract

An important enhancer/MAR (matrix attachment region) regulatory element in the mouse *tyrosinase* locus, located 15 kb upstream of the transcription initiation site, is responsible for imparting high level, position-independent tyrosinase transgene expression in neural crest-derived melanocytes. As MARs often coincide with the domain boundaries of gene loci, this MAR may also be the 5' domain boundary of the *tyrosinase* locus. General DNase I sensitivity assays of genomic DNA from cultured Cloudman S91 mouse melanoma cells showed that the region immediately flanking the upstream side of the MAR demonstrates a sensitivity approaching that of bulk chromatin, indicating that the MAR may be acting as a structural transition point between the open chromatin structure of the *tyrosinase* gene and the more closed architecture synonymous with heterochromatin. Four novel melanocyte-specific DNase I hypersensitive sites (DHS) were mapped in the human *tyrosinase* gene locus of cultured SK-Mel-28 human melanoma cells. Three DHS were found upstream of the transcription start site: one at -10.5 kb which may represent the human homologue to the -15 kb enhancer/MAR in the mouse *tyrosinase* locus, one co-localizing with the promoter, and one representing a -2 kb enhancer element that is essential for high level human *tyrosinase* expression. A DHS located 15 kb downstream of exon 5 was also found that maps to no known *cis*-regulatory element. In addition, transient transfection analyses using recombinant *luciferase* reporter genes showed that the human *tyrosinase* coding sequences exhibit repressor activity, an observation that might explain discrepancies of coat pigmentation of transgenic mice generated in different studies. As well, the SK-N-SH human neuroblastoma cell line was used to investigate the temporal activation of the human *tyrosinase* gene locus.
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List of Abbreviations

α-MSH  α-melanocyte stimulating hormone
Abf1  autonomous replication sequence binding factor 1
atRA  all trans-retinoic acid
bp  base pair(s)
BEAF  boundary element-associated factor
bHLH  basic helix-loop-helix
bHLH-Zip  basic helix-loop-helix-leucine-zipper
BSA  bovine serum albumin
cAMP  3',5'-cyclic adenosine monophosphate
CAT  chloramphenicol acetyl transferase
c<sup>m</sup>  <i>chinchilla-mottled</i> mouse mutation
CPM  count(s) per minute
ddH<sub>2</sub>O  distilled, deionized water
DEPC  diethyl pyrocarbonate
DHS  DNase I hypersensitive site(s)
DNase I  Deoxyribonuclease I
DOPA  3,4-dihydroxyphenylalanine
DHI  5,6-dihydroxyindole
DHICA  5,6-dihydroxyindole-2-carboxylic acid
EDTA  ethylene diamine tetraacetic acid
g  force of gravity
<table>
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<tr>
<td>GST-P</td>
<td>glutathione transferase P</td>
</tr>
<tr>
<td>HMG</td>
<td>high mobility group</td>
</tr>
<tr>
<td>htyr</td>
<td>human tyrosinase</td>
</tr>
<tr>
<td>Inr</td>
<td>initiator</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase(s)</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>LINE</td>
<td>long interspersed nucleotide repeat element</td>
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<tr>
<td>L1</td>
<td>long interspersed sequence one</td>
</tr>
<tr>
<td>L1Md</td>
<td>long interspersed sequence one: <em>Muscularis domesticus</em></td>
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<td>MAR</td>
<td>matrix attachment region</td>
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<tr>
<td>MCS</td>
<td>multiple cloning site</td>
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<tr>
<td>Mi</td>
<td>microphthalmia protein</td>
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<tr>
<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MLL</td>
<td>mixed lineage leukemia</td>
</tr>
<tr>
<td>MNase</td>
<td>micrococcal nuclease</td>
</tr>
<tr>
<td>mtyr</td>
<td>mouse tyrosinase</td>
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<tr>
<td>NF1</td>
<td>nuclear factor 1</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κB</td>
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<tr>
<td>NURF</td>
<td>nucleosome remodeling factor</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>Rap1</td>
<td>repressor activator protein 1</td>
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<tr>
<td>RBP-Jκ</td>
<td>recombination signal sequence binding protein-Jκ</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>RLB</td>
<td>reporter lysis buffer</td>
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<td>RPE</td>
<td>retinal pigment epithelium</td>
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<td>S/MAR</td>
<td>scaffold/matrix attachment region</td>
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<tr>
<td>scs</td>
<td>specialized chromatin structure</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride/sodium citrate</td>
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<td>TBE</td>
<td>Tris/borate/EDTA</td>
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<td>TE</td>
<td>Tris/EDTA</td>
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<td><em>tyrosinase</em> element-1</td>
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<tr>
<td>TFIID</td>
<td>transcription factor IID</td>
</tr>
<tr>
<td>Th1</td>
<td>Thing 1</td>
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<td>TRP</td>
<td>tyrosinase-related protein</td>
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<tr>
<td>TRS</td>
<td><em>tyrosinase</em>-related segment</td>
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<td>U</td>
<td>units</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulatory factor</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
<tr>
<td>yp-1</td>
<td><em>yolk protein</em>-1</td>
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Acknowledgements

To my wife, Anna, I owe everything. Though her patience has been tested through the years, she has never stopped believing in me. She has given much of herself so that I could accomplish this goal and for that I am eternally indebted to her.

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1. Experimental Objectives

Important regulatory elements exist in the upstream regions of the mouse tyrosinase gene that are essential for position-independent, high level expression of tyrosinase transgenes (Porter and Meyer, 1994; Ganss et al., 1994a). This region, demonstrated in vitro as a strong DNase I hypersensitive site (DHS) 15 kilobases (kb) upstream of the transcription start site (Porter et al. 1991; subsequently mapped as 12 kb in Ganss et al., 1994) possesses in vitro matrix attachment properties and harbours elements allowing for enhanced tyrosinase expression by both neural crest-derived melanocytes and retinal pigment epithelial (RPE) cells derived from the neuroectoderm. In addition, this region contains a silencer element that inhibits tyrosinase expression by the RPE. Matrix attachment regions (MARs) are believed to be associated with some gene domain boundaries (Phi-Van and Strättling, 1988; Levy-Wilson et al., 1989). Therefore, one objective of this study was to determine whether this MAR is the upstream domain boundary of the tyrosinase gene locus. By performing general DNase I sensitivity assays of the genomic DNA flanking the mouse enhancer/MAR and by establishing this MAR as the upstream boundary element, it could be concluded that all of the cis-acting regulatory sequences required for tyrosinase expression would likely reside at or downstream of the MAR.

Another objective was to determine whether and where a homologous enhancer/MAR resides in the human tyrosinase gene locus by searching for tyrosinase-specific DHS in genomic DNA from tyrosinase-expressing cultured human melanoma cells. Because vital regulatory elements are often conserved between species, discovery of a human homologue would lend credence to the importance of this region in tyrosinase gene regulation. Also, the subsequent isolation and subcloning of the hypersensitive region suspected of harbouring the
enhancer/MAR would enable the study of this region's role in human tyrosinase expression and would provide further insight into the regulatory similarities and differences that exist in tyrosinase expression between mice and humans.

As an adjunct to searching for the homologous human tyrosinase enhancer/MAR, an additional goal was to establish the temporal relationship between chromatin structural changes within the human tyrosinase locus and the expression of tyrosinase enzyme. This was to be done by assessing the DNase I hypersensitivity patterns that are established within the tyrosinase locus of differentiated cells of a human neuroblastoma cell line, a potential model for neural crest development, differentiation and maturation.

2. Background

2.1. Chromatin Structure and its Influence on Gene Expression

Chromatin structure plays an integral part in the control of gene expression (Felsenfeld, 1992; Wallrath et al., 1994). For tissue-specific, temporally correct and high level expression of a gene, it is imperative that trans-acting transcription regulatory factors have access at the appropriate times during cell development to their associated cis-acting promoter and enhancer DNA binding sites. The structural organization of the chromatin associated with a gene determines whether these interactions are possible. The complex, hierarchical structural organization of eukaryotic chromatin imposes substantial physical constraints on the interaction between DNA and regulatory proteins and tends to suppress transcriptional activation as a result (Felsenfeld, 1992). That transcription can occur indicates that these repressive obstacles can be removed or minimized through the alteration of the chromatin structure of a gene in a cell-specific and developmentally correct fashion.
2.1.1. Chromatin Structure

To fit within the confines of the nucleus, eukaryotic DNA experiences several distinct levels of organization that cause upwards of 1000-fold compaction of the chromatin. The nucleosome is the fundamental chromatin repeating subunit and consists of approximately 200 base pairs (bp) of DNA: an invariant 146 bp wound twice around a basic histone protein octamer core and, depending on the organism, a variable length of inter-histone linking DNA bound by the linker histone H1 or H5 variant (van Holde, 1988). Histone H1 binding helps to condense the chromatin and is believed to play a role in the repression of transcriptional activity as shown by the reduced numbers of histone H1 molecules in regions harbouring active genes or those poised to be expressed (Tazi and Bird, 1990; Käs et al., 1993). Coiling the nucleosome thread into a 30 nm solenoid is believed to form the basic structure of both interphase chromatin and mitotic chromosomes. Further higher order packaging is facilitated by non-histone proteins which anchor the chromatin to a proteinaceous nuclear matrix or scaffolding via specific DNA sequences termed ‘scaffold’ or ‘matrix attachment regions’ (S/MARs) forming structurally delimiting loops that are on average approximately 30 to 90 kb in length (Mirkovitch et al., 1984; Gasser and Laemmli, 1986). These structural domains may offer another level of high order compaction of the DNA (Gasser and Laemmli, 1986) facilitating the packaging of chromatin within the confines of the nucleus.

MARs are regions of A/T-rich DNA that bind a nuclear scaffolding composed largely of nuclear laminins via proteins that do not recognize specific DNA sequences but rather the particular structural features that are induced in the DNA in these A/T-rich areas. Although the identity of the proteins that anchor the DNA to the nuclear matrix via MARs in vivo is unknown, topoisomerase II is a good candidate as it can bind the narrow minor groove
structure of A/T-rich DNA with relatively high affinity as shown by the selective inhibition of MAR-protein interactions in vitro by distamycin A, an antibiotic that also recognizes the minor groove structure of A/T-rich DNA (Adachi et al., 1989; Käs et al., 1989). Topoisomerase II, essential for chromosome condensation and segregation (Cockerill and Garrard, 1986; Zhao et al., 1993), has binding sites in MAR sequences (Cockerill and Garrard, 1986) and has been isolated from extracted nuclear matrices (Berrios et al., 1985) and chromosomal scaffolding (Earnshaw et al., 1985). Histone H1 also binds MAR (or MAR-like) sequences in vitro and can be selectively displaced by distamycin (Käs et al., 1989). A model for the localized opening of the condensed chromatin structure that precedes gene expression has been proposed by Käs et al. (1993) and involves the redistribution of histone H1 from the inter-nucleosome linker DNA and a subsequent increase of DNA accessibility as a result of the displacement of histone H1 proteins from the MAR sequences by an in vivo distamycin analogue. Zhao et al. (1993) found that high mobility group protein (HMG)-I and its isoform HMG-Y, which bind A/T-rich sequences via an ‘AT-hook’ motif, may act as in vivo distamycin analogues alleviating the histone H1-mediated repression of gene activity.

The presence of ‘AT-hook’ DNA-binding domains can be found as well on transcription factors such as the mixed lineage leukemia (MLL) protein, the gene of which is translocated in many acute lymphoid and myeloid leukemias (Broeker et al., 1996). Separate from the putative activator and repressor domains, the ‘AT-hook’ domain of the MLL protein has been shown to bind MAR sequences in vitro (Broeker et al., 1996) and may allow for nuclear localization of the MLL protein, or MLL-fusion proteins, such that they can exert their function within the nucleus (Joh et al., 1997). Sequences rich in A/T-tracts may act as
targets for therapeutic DNA-binding drugs such as distamycin and microgonotropin (Chiang et al., 1997) which may effectively inhibit nuclear localization or transcription factor-DNA complex formation, down-regulating gene expression of tumour cells in the process.

The organization of MARs appears to be non-random. Mirkovitch et al. (1984) found that A/T-rich nuclear matrix binding sites are present in the non-transcribed intergenic regions of the Drosophila histone and major heat shock protein gene clusters defining the bases of chromatin looped domains. MARs frequently co-localize with the transcriptional enhancers of genes (Mirkovitch et al., 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986, Jarman and Higgs, 1988; Blasquez et al., 1989; Jenuwein et al., 1997) including the mouse tyrosinase gene (Porter and Meyer, 1994) and coincide with origins of replication (reviewed in Roberge and Gasser, 1992 and Boulikas, 1993). MARs also appear to punctuate the boundaries of many gene domains, delimiting independent transcriptional units.

2.1.2. Genes are Organized into Topologically Defined Domains

The immense size of the higher eukaryotic cell’s genome and the vast amounts of genetic information contained within it would seem to pose a substantial risk of regulatory chaos when it comes to controlling gene expression. Given the ability of enhancers to exert their effects over long distances (Jenuwein et al., 1993), cis-regulatory elements of one locus could conceivably influence the transcriptional activity of genes in nearby loci. However, the compartmentalization of genes into isolated, higher order topological domains is believed to facilitate the complex task of regulating gene transcription in an orderly fashion (Dillon and Grosveld, 1994). The concept of a gene domain states that for any given gene or series
of related genes, the coding regions and all associated cis-regulatory elements controlling the expression of that gene or genes are sequestered in a discrete region of open chromatin representing a functional unit (Phi-Van and Strätling, 1988; Levy-Wilson and Fortier, 1989). These domains are thought to be defined by boundary elements that may prevent the various enhancer elements found within those boundaries from acting on genes in adjacent loci, limiting the enhancer activity to the affiliated gene within the same domain (Kellum and Schedl, 1992; Cai and Levine, 1995). As well, boundary elements may help insulate the internal sequences from the interfering influences of regulatory elements associated with flanking active genes (Esseinberg and Elgin, 1991; Cai and Levine, 1995) and from both positive and negative position effects mediated by the surrounding inactive chromatin structure (Stief et al., 1989; Kellum and Schedl, 1991; Felsenfeld, 1992).

The mouse tyrosinase upstream MAR identified by Porter et al. (1991) displays some characteristics of known boundary elements and may represent the 5' tyrosinase domain boundary. The mouse tyrosinase upstream enhancer/MAR elements and the investigations which have resulted in our present understanding of how they control tyrosinase gene expression will be discussed in the following sections. A synopsis of some of the elements that are believed to provide boundary function and to help determine the chromatin structure of other gene loci will set the stage for a more in-depth examination of similar elements found in the tyrosinase locus.

2.1.2.1. Domain Boundaries: Characteristics, Location and Function

Boundary elements generally fall into one of two classes: those that demonstrate an affinity for nuclear matrices and those that do not. In either case, one criterion that has been
used to define boundary elements is that they delimit the decondensed, nuclease-sensitive chromatin of an active domain from the compacted, relatively nuclease-insensitive flanking chromatin outside of the domain (Dillon and Grosveld, 1994). Chromatin unfolding may occur in part through the reduction of histone H1 occupancy of the DNA linker regions between the nucleosomes (Käs et al., 1993) allowing for increased access by the DNase I nuclease. Although not all insulator elements are necessarily boundary elements - the gypsy retroposon in Drosophila is one example that appears not to be (Roseman et al., 1993) - another criterion that is used to help define boundary elements is their ability to protect transgenes from position effects.

The specialized chromatin structures (scs and scs') found at the proximal and distal boundaries of the divergently expressed Drosophila 87A7 hsp70 heat shock genes act as putative gene domain boundary elements in that they delimit an approximately 12 kb domain of DNase I- and micrococcal nuclease (MNase)-sensitive chromatin of expressing cells (Wu et al., 1979; Hans et al., 1985). They do not attach to the nuclear matrix and so are not considered MARs, although, as their appearance as constitutive DHS (Udvardy et al., 1985) alludes, they do bind nucleoproteins in vivo; two that have been isolated (boundary element-associated factor (BEAF)-1 & BEAF-2) contribute to the buffering effect of the scs’ element (Zhao et al., 1995; Hart et al., 1997). The scs and scs’ elements promote position-independent transgene expression when they flank both ends of a gene and its associated regulatory elements (Kellum and Schedl, 1991). In transgenic studies, Kellum and Schedl (1991) showed that most of the time, flanking scs elements are able to protect the white gene, responsible for conferring the wild-type red eye phenotype on Drosophila, from both positive and negative position effects whereas considerable position effect was observed in transgenic
flies that contained reporter genes not flanked by scs elements causing reduced levels of expression. A subsequent study demonstrated the ability of both the scs and scs' elements to prevent the yolk protein-1 enhancer element (yp-1) from interacting with the hsp70 promoter fused with a lacZ reporter gene when either one was placed between yp-1 and the promoter, effectively eliminating the β-galactosidase expression of transgenic flies (Kellum and Schedl, 1992). The same insulative properties were not seen when the scs elements were placed immediately upstream of the yp-1 enhancer suggesting that the scs elements position the enhancer and promoters in different structural compartments when placed between them, hence, chromatin compaction may inhibit mutual interaction (Kellum and Schedl, 1992). Cai and Levine (1995) observed that the scs elements exert their buffering effects in a directional manner, blocking the interaction of a proximal enhancer with a distally placed promoter and only allowing interplay between the proximal enhancer and an adjacent promoter on the same side as the enhancer. That chromatin structure is involved in the enhancer-blocking mechanism is also supported by the observation that the scs elements do not insulate enhancer-promoter interaction in transient expression assays (Hart et al., 1997). In this light, it is interesting that the scs boundary elements have been found to buffer the high torsional tension experienced by the non-transcribed gene sequences within the boundaries, leading to the unrestrained negative supercoiling characteristic of active genes (Cockerill and Garrard, 1986), from the relaxed flanking sequences outside the boundaries (Jupe et al., 1993 & 1995).

MARs have been mapped to the putative domain boundaries of many genes, including the chicken ovalbumin gene (Lawson et al., 1982) the glyceraldehyde-3-phosphate dehydrogenase gene (Alevy et al., 1984), the chicken lysozyme gene (Jantzen et al., 1986;
Strätling and Dölle, 1986; Phi-Van and Strätling, 1988), and the human apolipoprotein B gene (Levy-Wilson and Fortier, 1989). In each case, the MAR boundary elements define the limits of a DNase I-sensitive domain of cells that express the respective genes. The MARs representing the domain boundaries of the chicken lysozyme gene, which is expressed in the oviduct and in the myeloid lineage of the hematopoietic system, are known as A-elements and have been shown to insulate reporter transgenes against position effects of stably transfected cultured promacrophage cells (Stief et al., 1989; Bonifer et al., 1991) but not of macrophages of transgenic mice (Bonifer et al., 1994). As the A-elements demonstrated no enhancer activity in the transient expression assay, the increased level of transgene expression by stably transfected promacrophage cells was most likely due to the increased number of the integrated transgenes that were able to be expressed as a result of being liberated from position effects.

In transgenic mice harbouring constructs containing the same elements in context with the chicken lysozyme gene, the A-elements functioned by reducing the degree of ectopic expression of the transgene, perhaps insulating the transgene from spurious activation by closely positioned non-lysozyme gene regulatory elements (Bonifer et al., 1994). Position-independent expression was observed in macrophages when all the known cis-acting regulatory elements were included in the constructs regardless of whether the A-elements were present. In cultured cell systems, cells containing the transgenes are selected for on the basis of the expression of a linked marker gene (Philipsen et al., 1993) and as a result, the integrated transgenes are more likely to find themselves in active chromatin and be more readily expressed. Mouse transgenes integrate into the genome early during development, prior to the formation of heterochromatin (Reitman et al., 1993), and are less apt to find
themselves in a transcriptionally favourable region upon cell development and differentiation. Although the A-elements may be able to protect a transgene from negative position effects in relatively favourable chromatin environments, it appears that they may not be able to perform the same task in more transcriptionally suppressive surroundings. The enhancer and promoter elements, when working cooperatively, foster position-independent, tissue-specific, high level lysozyme expression. Together, these elements demonstrate Locus Control Region (LCR)-like activity.

2.1.3. Locus Control Regions and Transcriptional Activation

LCRs provide dominant control of gene expression activation causing enhanced, tissue-specific expression of associated genes independent of their position along the genome. The 'classic' LCR, as found in the \( \beta \)-globin gene locus of many mammalian species (Grosveld \textit{et al.}, 1987; reviewed in Hardison \textit{et al.}, 1997), is a collection of DNA sequences, usually marked by DHS in \( \beta \)-globin gene expressing cells, that can bind a variety of \textit{trans}-acting regulatory factors. These proteins can interact with an associated promoter preventing histone binding at the promoter and rendering the region DNase I hypersensitive and accessible by \textit{trans}-acting transcription factors (Felsenfeld, 1992; Reitman \textit{et al.}, 1993). The mechanism underlying an LCR's ability to control chromatin structure over long distances is poorly understood but it is thought that the LCR opens the chromatin in a tissue-specific manner, possibly through the conscription of chromatin remodeling enzymes such as the SWI/SNF (Richard-Foy, 1994) or nucleosome remodeling factor complexes (NURF, Tsukiyama and Wu, 1995), and prevents higher order chromatin structure from inactivating the gene domain (Reitman \textit{et al.}, 1993). The human \( \beta \)-globin LCR, located in the far
upstream region of the locus, controls an over 100 kb domain containing five erythroid-specific globin genes, each of which is expressed at different times during development (Forrester et al., 1990). In erythroid cells, the entire domain is sensitive to DNase I relative to chromatin that is transcriptionally inactive and exhibits regions of DNase I hypersensitivity that map to enhancer elements in the LCR and the promoter regions for each gene. As well, the genes replicate early during S phase. The chromatin restructuring resulting in increased general DNase I sensitivity and hypersensitivity occurs prior to transcription initiation of the globin genes (Blom van Assendelft et al., 1989). When the LCR is deleted, as in Hispanic (γδβ) thalassemia, transcriptional activation of the cis-linked genes is absent and the entire β-globin domain becomes DNase I-resistant and replicates late during S phase, the latter two phenomena being consistent with heterochromatin (Forrester et al., 1990). The human β-globin LCR was the first such region to be identified and remains the most exhaustively studied, but similar functioning regions have been found associated with other gene loci (Greaves et al., 1989; Chamberlain et al., 1991; Aronow et al., 1992; Neznanov et al., 1993; Bonifer et al., 1994).

The β-globin LCR is comprised of individual and separable components that are able to perform some LCR regulatory functions independently (Morely et al., 1992; Hug et al., 1996) although all of the elements must be present for maximal gene expression. Several MARs have been mapped to the human β-globin locus, two of which co-exist with the boundaries of the functional domain (Jarman and Higgs, 1988). No insulative capacity has been attributed to the boundary MARs however, and their role in LCR functioning, if any, is not yet known.

The upstream enhancer/MAR of the mouse tyrosinase gene has separable enhancer and
matrix attachment elements and exhibits LCR-like activity. Trangenes containing the entire mouse tyrosinase upstream enhancer/MAR region are able to be expressed at high levels, largely independently of their position within the genome. When the enhancer/MAR is removed from its natural genomic position, the tyrosinase gene locus becomes condensed and transcriptionally inactive (Porter et al., 1991).

2.2. Tyrosinase

The biosynthesis of melanin pigment in mammalian melanocytes occurs, in part, through the action of the tyrosinase enzyme (monophenol, L-dopa: oxygen oxidoreductase, EC 1.14.18.1). Tyrosinase is a copper-containing glycoprotein that is essential for catalysis of the first two rate-limiting steps that lead to the synthesis of melanin: the hydroxylation of the amino acid tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the subsequent oxidation of DOPA to DOPA quinone (see Figure 1). Tyrosinase also has been shown to catalyze the conversion of 5,6-hydroxyindole (DHI) to melanochrome (Korner and Pawelek, 1982). However, the importance of tyrosinase in this regard is unclear as not only do 5,6-hydroxyindoles have a propensity to oxidize, thus reducing the need for a highly specific facilitating enzyme, but peroxidase has been found to be far more effective than tyrosinase for the catalysis the conversion of DHI to melanochrome under similar conditions (Prota, 1992). Regardless, the importance of tyrosinase to melanogenesis is clear: in the absence of functional tyrosinase, melanin is not synthesized and the albino phenotype prevails irrespective of genotype (Silvers, 1979). While melanin pigment is not essential for life (Montagna et al., 1993) it is required for proper neuronal migration along the visual pathways during development and, as well, provides cells with vital protection against the
Figure 1. Mason-Raper Pathway for Melanin Biosynthesis. Adapted from Winder et al., 1994.
damaging effects of ultraviolet radiation (Spritz, 1994). Melanin and its precursors (e.g., DHI and DHICA) are effective antioxidants and as such are well suited to scavenge potentially harmful oxygen free-radicals that are generated in cells upon their exposure to ultraviolet radiation (Montagna et al., 1993). Decreased melanin levels due to the absence or dysfunction of tyrosinase during foetal development causes severe visual disturbances during later life such as nystagmus, lack of binocular vision, and decreased visual acuity (Barton et al., 1988) and predisposes the individual to various types of skin neoplasms, the most notable being malignant melanoma (Spritz, 1994).

2.2.1. Melanocytes and Melanogenesis

Normally, tyrosinase synthesis is restricted to melanocytes of which there are two types: those derived from neural crest tissue and those derived from the neural tube. Neural crest-derived melanocytes are dendritic cells that in humans reside at the dermal/epidermal junction, in the iris stroma and choroid of the eye, the inner ear and in the hair follicles. In mice, this same type of melanocyte resides predominantly at the base of hair follicles, in the lacrimal Harderian gland, in the anterior iris stroma and choroid, and in the dermis of the tail and ears. Pigmented cells are found in the RPE and posterior iris in both humans and mice. These non-dendritic melanocytes are derived from the outer layer and the ciliary margin, respectively, of the optic cup which originates from the neural tube during development (Jackson, 1994). Both Schwann cells (Garcia and Szabo, 1979) and Schwannommas (Killeen, 1988) have been documented to contain melanosomes and be pigmented; however, this may reflect the existence of a common bipotent progenitor cell for melanocytes and Schwann cells (Tsokos et al., 1985).
Melanin synthesis occurs in special membrane-bound vesicles known as melanosomes which are unique to cells of the melanocyte lineage. Tyrosinase is incorporated into the vesicle membrane at its early premelanosomal stage (Montagna et al., 1993) and works in concert with other melanocyte-specific enzymes to catalyze the synthesis of two main classes of melanin pigments: eumelanin and pheomelanin (Hearing and Jiménez, 1987). Eumelanin is an insoluble, dark brown to black pigment and pheomelanin is an alkali-soluble yellow to reddish-brown pigment containing sulfur (Montagna et al., 1993). As tyrosine is polymerized to melanin it is deposited on a filamentous protein scaffolding in the premelanosome. The premelanosome eventually matures into a melanosome (Jiménez et al., 1988) which, in the case of the dendritic melanocytes, is transferred to the surrounding keratinocytes and hair-shafts by exocytosis. In contrast, the ocular melanocytes are continent and do not transfer the melanosomes to other cells (Garcia et al., 1979).

What type and how much melanin is synthesized is modulated by a myriad of factors. More than 60 gene loci influence melanogenesis (Kobayashi et al., 1994) by affecting melanocyte development, migration, and morphology as well as melanocyte-specific gene expression and melanosomal structure and function (reviewed in Jackson, 1994). Within the Mason-Raper melanogenic pathway (Figure 1), tyrosinase works intimately with two structurally similar yet catalytically distinct melanocyte-specific enzymes also directed to the premelanosome: Tyrosinase-Related-Protein (TRP)-1 and TRP-2. TRP-1 maps to the brown locus in mice (Jackson, 1988) and oxidizes DHICA (Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994) while TRP-2, located at the slaty locus (Jackson, 1992), acts as DOPAchrome tautomerase and catalyzes the conversion of DOPAchrome to DHICA (Tsukamoto et al., 1992). These three enzymes are commonly grouped into one family
given their structural similarities and coordinated expression (Kuzumaki et al., 1993) and activity (Kameyama et al., 1993).

2.2.2. The Tyrosinase Gene Locus

The structural gene for tyrosinase maps to the albino(c) locus on chromosome 7 in mice (Kwon et al., 1987; Müller et al., 1988; Beermann et al., 1990) and chromosome 11 (q14 > q21) in humans (Barton et al., 1988). The gene consists of five exons spanning 80 kb in the mouse (Ruppert et al., 1988) and the same number of exons spanning more than 50 kb in humans (Giebel et al., 1991). On the short arm of human chromosome 11 near the centomere (11.2 > cen.) there is a tyrosinase-related segment (TRS) consisting of exons 4 and 5 with intervening intronic DNA and an unknown length of 3' flanking DNA (Barton et al., 1988; Giebel et al., 1991). The TRS coding region sequence differs from the 11q tyrosinase gene by only 0.75% and in the non-coding regions by 2.6% and may represent a truncated and translocated tyrosinase pseudogene (Geibel et al., 1991).

The classical albino phenotype of mice is thought to result from a conserved cysteine to serine point mutation in the tyrosinase coding sequences (Yokoyama et al., 1990). Although mice with this mutation have a full complement of melanocytes and express tyrosinase to the same levels as the wild phenotype, the tyrosinase protein is non-functional and the melanocytes are amelanotic (Silvers, 1979). Over 50 mutations in the human tyrosinase locus are known to cause tyrosinase-deficient (Type I oculocutaneous) albinism with no one mutation being predominant (Spritz, 1994).
2.2.3. Regulation of Tyrosinase Gene Expression

Studies of the regulation of tyrosinase expression among vertebrates have revealed regions of striking structural and functional similarity as well as cis-acting control elements displaying distinct differences that reside in the promoter and proximal upstream regions of the tyrosinase gene (reviewed in Ferguson and Kidson, 1997). The evolutionary conservation of both the relative position and nucleotide sequence of many of these regulatory elements is indicative of their functional importance for controlling tyrosinase expression. As the regulatory components of the tyrosinase locus are elucidated and the regulatory scheme unfolds, it is evident that the transcription control process is complex. Efficient melanocyte-specific tyrosinase gene activation during development requires the concerted interaction of no fewer than three positive and one negative promoter cis-regulatory elements (Bentley et al., 1994) and tyrosinase modulation, ultimately affecting melanogenesis, is influenced by a variety of enhancer and silencer elements bound to transcription factors that are, in some cases, regulated by external stimuli. An upstream enhancer/MAR element appears to control the chromatin structure of the entire mouse tyrosinase locus and is essential for allowing both active transcription and effective control of the process (see Porter et al., 1991; Ganss et al., 1994a; Porter and Meyer, 1994; Montoliu et al., 1996; Porter et al., unpublished).

2.2.3.1. The Tyrosinase Promoter

The 270 bp of the mouse tyrosinase sequence immediately 5' of the transcription start site are sufficient to drive the expression of a CAT (chloramphenicol acetyltransferase) reporter gene in tyrosinase-positive human and mouse melanoma cells but not in tyrosinase-
negative melanoma cells or fibroblasts (Klüppel et al., 1991). Transgenic mouse studies showed that the same 270 bp region, in context of a minigene, is sufficient to direct the expression of mouse tyrosinase of both neural crest-derived melanocytes and those of the RPE (Klüppel et al., 1991) in a fashion temporally consistent with that of the endogenous mouse tyrosinase gene. Tyrosinase expression is detectable in the RPE at day 10.5 of gestation and in the hair follicle at day 16.5 and precedes the visible deposition of melanin in each cell-type (Beermann et al., 1992). These observations strongly suggest that all of the elements necessary for melanocyte-specific and developmental-stage-correct tyrosinase expression are found in this region of mouse promoter DNA. Transient transfection analysis using various lengths of human tyrosinase promoter sequences fused to a CAT reporter gene showed that only 115 bp of sequences 5' of the transcription start site were sufficient to drive the cell-specific expression of CAT in B16 mouse melanoma cells but not in JEG3 human choriocarcinoma (tyrosinase-negative) cells (Bentley et al., 1994). Common to this 115 bp region of both the mouse and human gene are three positive elements that are essential for efficient tyrosinase promoter function: the 11 bp M box containing an E-box motif (CATGTG), an SP-1 binding sequence, and a second E-box motif, overlapping with a non-consensus octamer element that binds the POU domain transcription factors Oct-1 and Brn-2/N-Oct3 (Eisen et al., 1995), associated with the initiator (Inr) region (Bentley et al., 1994). With the assistance of SP-1 (Kaufman and Smale, 1994), it has been speculated that the Inr may bind TFIID and the basal transcription machinery allowing for the initiation of transcription in the absence of a canonical TATA box, as is the case for the tyrosinase gene (Bentley et al., 1994). The E-box consensus sequence (CANNTG) is found in other genes and binds transcription factors with a basic helix-loop-helix-leucine-zipper (bHLH-Zip) motif.
that may be ubiquitous, such as USF (upstream stimulatory factor), or tissue-specific. The microphthalmia protein, *Mi* of mice and the microphthalmia-associated transcription factor (*MITF*) of humans, is a member of the bHLH-Zip family and is believed to be a melanocyte-specific regulatory factor protein that binds the *tyrosinase* M box and E-box elements *in vivo* and activates the *tyrosinase* promoter (Bentley *et al.*, 1994; Yasumoto *et al.*, 1994 & 1995).

The phenotypes resulting from mutations in the *microphthalmia* gene reflect the importance that *Mi* or *MITF* have in proper melanocyte differentiation and tyrosinase function. Homozygous null mutations in *Mi* present with small, poorly developed eyes and a complete absence of all melanocytes. Humans harbouring heterozygous mutations of *MITF* suffer from the dominantly inherited Waardenburg’s syndrome type II affecting both pigmentation and hearing (Moore, 1995). *In vitro* studies have shown that *Mi* can transactivate the human *tyrosinase* promoter (Bentley *et al.*, 1994) and that *MITF* can transactivate both the human and mouse *tyrosinase* genes (Yasumoto *et al.*, 1994 & 1995).

### 2.2.3.2. The *Tyrosinase* Proximal Enhancer

The human *tyrosinase* promoter region equivalent to the 270 bp mouse promoter that has been shown to direct melanocyte-specific expression appears to be weaker than the mouse promoter (Shibata *et al.*, 1992). The presence of a melanocyte-specific enhancer element (Ponnazhagen and Kwon, 1992; Ganss *et al.*, 1994b), termed *tyrosinase* element-1 (TE-1), 236 bp upstream of the transcription start site of the mouse promoter region but not of the human promoter may account for the observed differences. The human promoter’s melanocyte-specific activity, in transient expression studies, is augmented by a 200 bp enhancer region, the *tyrosinase* distal element (TDE), located between 1.8 and 2.0 kb
upstream of the transcription start site (Shibata et al., 1992; Ponnazhagen et al., 1994). Despite this region having substantial nucleotide homology with the same region of the mouse locus, functional analysis of the equivalent 200 bp region of the mouse did not enhance expression of an SV40 early promoter driven luciferase reporter gene transfected into MeWo mouse melanoma cells (Shibata et al., 1992). Two CATGTG E-box motifs are located in the TDE, one of which resides within the 39 bp core element, determined through deletional analysis to be the minimal sequence required to enhance reporter gene expression under control of the human tyrosinase promoter in melanoma cells (Shibata et al., 1992). The TDE is able to bind MITF in vitro and direct the melanocyte-specific activation of a reporter gene linked to the tyrosinase gene promoter (Yasumato et al., 1994).

2.2.3.3. Long Range Control of Tyrosinase Expression

Early investigations of the mouse tyrosinase locus involved generation of transgenic albino mice harbouring tyrosinase minigenes composed of mouse tyrosinase cDNA coding region and various lengths of upstream mouse tyrosinase genomic sequences to determine which regions contained vital regulatory elements (Beermann et al., 1990; Tanaka et al., 1990; Yokoyama et al., 1990). The transgenic mice resulting from these studies had variable pigmentation levels that did not correlate with transgene copy number and some transgenic lines showed mottled patterns. Both observations suggested position effects whereby the expression of the transgene is variably suppressed upon integration into transcriptionally inactive heterochromatin and pointed to the likelihood that the minigenes were lacking an important regulatory element that could prevent such effects.

Initial clues to the existence of a distal, upstream regulatory element that could control
the chromatin structure of the mouse *tyrosinase* locus and perhaps protect the gene from position effects were provided by Porter *et al.* (1991) during their investigation into the structure and effects of the *chinchilla-mottled* mouse mutation (*c*<sup>n</sup>). In its homozygous form, the *c*<sup>n</sup> mutation causes a clonal variation of *tyrosinase* expression by melanocytes that can be visualized as a transverse light- and dark-grey stripe pattern (Porter *et al.*, 1991; Jackson, 1994). A rearrangement resulting in the separation of no less than 30 kb of distal upstream *tyrosinase* DNA - starting 5 kb upstream of the transcription start site - containing a MAR from the promoter and coding sequences is believed to be responsible for the characteristic mosaic pigmentation pattern. At the wild-type locus, DHS are present at the promoter and 15 kb upstream of the transcription start site which co-localizes with a MAR. Porter *et al.* (1991) observed that light melanocyte clones had no DHS at the promoter, had a weak DHS at the relocated region equivalent to the -15 kb site and exhibited diminished *tyrosinase* expression from that of wild-type whereas the dark clones possessed both DHS and had lower *tyrosinase* expression than wild-type but more than the light clones. The general DNase I sensitivity of the coding region was indistinguishable between dark clones and wild-type cells whereas the coding region of light cells was of intermediate sensitivity between fully open and compacted chromatin. It appears that the -15 kb DHS/MAR region in the normal position is required to generate open chromatin sufficient to permit wild-type levels of *tyrosinase* expression and that the *c*<sup>n</sup> rearrangement renders the DHS/MAR region too distant to effect adequate structural alterations of the locus to allow this.

The dependence of position-independent transgene expression on upstream *tyrosinase* sequences was illustrated by the rescue of the albino phenotype through microinjection of a 250 kb yeast artificial chromosome (YAC) containing the mouse *tyrosinase* gene with 155 kb
of 5' flanking genomic DNA into albino mice (Schedl et al., 1993). The transgenes were expressed in a copy number-dependent fashion indicating that there were one or more regulatory elements included in the YAC that were not present in the tyrosinase minigenes previously used. One of these elements was likely the -15 kb enhancer/MAR identified by Porter et al. (1991) because subsequent studies by Porter and Meyer (1994) and Ganss et al. (1994a) showed that the enhancer/MAR sequences were required to impart position-independent, wild-type levels of transgene expression by melanocytes.

Porter and Meyer (1994), Montoliu et al. (1996) and Porter et al. (unpublished) generated transgenic albino mice containing human tyrosinase cDNA and the mouse tyrosinase promoter region linked to all, some or none of the enhancer/MAR region and found that the degree of pigmentation varied considerably among the different lines of mice depending on what part of the enhancer/MAR was included in the construct. What became clearly evident was that the mouse upstream enhancer/MAR demonstrated distinct regulatory properties with respect to tyrosinase expression by melanocytes from the two developmental lineages. The entire enhancer/MAR had the greatest effect on neural crest-derived melanocytes; in most cases, imparting high, sometimes wild-type, levels of position-independent transgene expression only by melanocytes of that lineage. Two transgenic mouse lines containing the entire enhancer/MAR displayed a mosaic coat pattern indicating position-effect variegation, suggesting that the entire upstream regulatory region was not sufficiently strong to protect against all negative position effects. Constructs containing the enhancer but lacking most of the MAR imparted high levels of transgene expression to neural crest-derived melanocytes but the expression was variable due to position effects and independent of copy number between lines. Melanocytes derived from the optic cup had
lower transcription levels than the neural crest-derived melanocytes from those transgenic mouse lines harbouring constructs that included the entire enhancer/MAR region or enhancer alone. In mice transgenic with constructs containing only the MAR sequences, the transgenes were protected to some extent against position effects in both neural crest- and neural tube-derived melanocytes but had expression levels far below those of wild-type in neural crest-derived melanocytes indicating no expression enhancement. Substantially enhanced expression was seen in RPE, however. Thus, the enhancer element appears to have a positive neural crest-specific effect and an RPE-specific silencing effect.

The -15 kb DHS co-localizes with the neural crest melanocyte-specific enhancer and, based on DNase I footprinting and gel-shift analysis, binds several proteins (Porter and Meyer, 1994; Ganss et al., 1994a). The enhancer region contains binding motifs resembling those for several known regulatory proteins: ER, NF-1, AP-4, AP-1, and Elk-1 (Ganss et al., 1994a; Porter et al., unpublished). Although both the MAR and enhancer elements are required for altering the chromatin structure of the mouse tyrosinase locus to facilitate transcription, at this time little is known about how these particular elements might accomplish this task.

3. Experimental Rationale

3.1. The Mouse Tyrosinase Locus: Defining the 5' Domain Boundary

Actively transcribed genes and those poised to be expressed are embedded in chromatin that displays a greater sensitivity to DNase I than transcriptionally inactive chromatin (Gross and Garrard, 1988). As shown by chromatin structural analysis of many genes, this enhanced sensitivity extends considerable distances beyond the transcribed sequences both
upstream and downstream of the gene and is delimited at the 5' and 3' ends by putative boundary elements. Past the boundaries, the general DNase I sensitivity decreases and the flanking DNA adopts a conformation that is consistent with heterochromatinized DNA: a highly condensed structure much more resistant to DNase I cutting \textit{in vitro} but more importantly from an \textit{in vivo} functional perspective, largely inaccessible by regulatory proteins. If the upstream enhancer/ MAR is the 5' mouse tyrosinase domain boundary, there should be distinct differences in the degree of DNase I sensitivity of the chromatin to either side of the enhancer/MAR. Identifying the upstream enhancer/MAR as the 5' domain boundary would, by definition, relegate all \textit{tyrosinase cis}-regulatory elements to and/or downstream of that boundary.

3.2. Mapping the Human Homologue to the Upstream Enhancer/MAR

Chromatin restructuring allowing \textit{trans}-regulatory transcription factors access to their cognate binding sites entails the establishment of nucleosome-free regions - nuclease hypersensitive sites - over promoter and enhancer sequences preceding or accompanying gene transcription (Gross and Garrard, 1988; Felsenfeld, 1992; Jenuwein \textit{et al.}, 1993). Determining the location of the nuclease hypersensitive sites within a transcriptionally competent gene locus is often the initial step for determining where the key regulatory elements that help control transcription reside within the locus. On that basis, the upstream and downstream regions of the human \textit{tyrosinase} locus were mapped for DHS primarily to locate a possible homologue to the mouse \textit{tyrosinase} upstream enhancer/MAR but also to find any other previously undocumented potential \textit{cis}-regulatory elements present in the human locus.
The importance of a regulatory element for controlling gene expression is reflected by the degree to which the nucleotide sequence and relative position of the element are conserved between species throughout evolution. The LCR of the \(\beta\)-globin gene cluster, necessary for establishing an open chromatin structure over the entire domain and helping to direct developmentally correct and enhanced globin protein expression, is positionally maintained in the 5' regions of the locus upstream of the \(\epsilon\)-globin gene in several mammalian species including mouse and humans (Moon and Ley, 1990; Chung et al., 1993; Hardison et al., 1997). At least four of the five nuclease hypersensitive sites associated with the LCR are present in both mice and humans and their relative positions are preserved. As well, significant nucleotide homology of the LCR exists within the DHS cores and adjacent 5' and 3' regions flanking the DHS suggesting functional conservation of these elements between species (Hardison et al. 1997). In the tyrosinase gene locus, the mouse upstream enhancer/MAR plays a vital role similar to that of the \(\beta\)-globin LCR in that it strongly influences the chromatin structure of the tyrosinase locus during melanocytic development to allow for tyrosinase expression. Thus, it would not be surprising to find a homologous element in a similar upstream location in the human tyrosinase locus. Indeed, the functional conservation of such a region or particular elements within this region would serve to emphasize the critical role that this region plays in the proper developmental regulation of tyrosinase transcription.

3.3. Developmental Activation of the Human Tyrosinase Gene

During development, the ability of cells to differentiate into other cell-types is progressively restricted until they become terminally differentiated and exhibit a unique
phenotype. For melanocytes, phenotypical uniqueness lies in their ability to express tyrosinase and to synthesize melanin. Pigmentation appears after cells of the melanocytic lineage have differentiated from the melanoblast but at what point the chromatin structural changes occur in the tyrosinase locus to poise the gene for active expression allowing for subsequent melanin synthesis is unknown. One of the principal controlling mechanisms of developmental gene expression involves the remodeling of the chromatin structure of a gene in a developmental stage-specific and cell-type-restricted manner. The timing of this remodeling is vital as the transcriptional status of a gene at a given developmental stage is contingent on the temporally correct generation of an active chromatin structure.

This is clearly evident with the chicken lysozyme gene where the activation of different enhancer elements during macrophage development dictates the stage at which the lysozyme gene is transcribed. Early during macrophage development, formation of DHS over the promoter and two early enhancers located at -3.9 and -6.1 kb drive low level lysozyme expression by myeloblasts (Huber et al., 1995). The lack of DHS formation at the early enhancers and the subsequent abolition of early lysozyme expression by transgenic mice carrying constructs with a deleted lysozyme promoter underlines the importance of promoter-enhancer interaction in order to stabilize transcription factor complexes and to activate chromatin structure over the early enhancer elements (Huber et al., 1997). The removal of the early -6.1 kb enhancer delays lysozyme gene activation until the more developmentally mature promacrophage stage (Jägle et al., 1997). Expression at this stage coincides with the formation of a DHS over the -2.7 kb late enhancer, allowing this region to interact with the promoter to drive transcription.

Roque et al. (1996) studied the chromatin structure of the 3' enhancer region of the
mouse immunoglobulin kappa (κ) gene in a variety of cell lines representing B-cells at various stages of developmental maturity. This enhancer region is required for control of tissue-specific and temporally-correct transcription of the gene. They identified two distinct states of chromatin structure associated with different stages of development: E (early) chromatin, found in non-κ expressing pro-, pre-, and mature B cells and L (late) chromatin found in plasma cells and correlating with immunoglobulin κ expression. In the E chromatin, two DHS were detected in the 120 bp core region while the L chromatin showed a substantial increase of DNase I hypersensitivity at the two DHS in the core region and an appearance of three additional DHS in the flanking sequences. Upon closer scrutiny of the enhancer element by ligation-mediated PCR-assisted genomic footprinting and MNase sensitivity analysis it was revealed that chromatin structural changes coincided with the differential binding of transcription factors as well as with the displacement of the nucleosomes in this region to accommodate them. Interestingly, one mature B cell line showed an intermediate hypersensitivity pattern between E and L chromatin with a corresponding low level of κ expression, possibly representing a transitional state of chromatin remodeling from a transcriptionally inactive to a fully active state.

The above two examples suggest that the developmentally regulated chromatin remodeling of the tyrosinase locus leading to the activation of tyrosinase expression might be observed by monitoring the temporal formation of the melanocyte-specific DHS and correlating the architectural changes with the amount of tyrosinase mRNA synthesized. It was hypothesized that such structural alterations could be demonstrated using the human SK-N-SH neuroblastoma cell line, a tumour line of the peripheral nervous system comprising cells of neural crest lineage that have the ability to differentiate along a committed
melanocytic cell pathway. Neuroblastoma cells appear to represent a relatively early, pluripotent stage of the neural crest as several human neuroblastoma cell lines have been shown to emulate the differentiation of the neural crest in vitro.

The SK-N-SH cell line is comprised of cell sub-types with distinct morphological and biochemical phenotypes: neuronal, Schwannian, and melanocytic. These cell sub-types have arisen from the common SK-N-SH parental source (Biedler et al., 1981; Sidell et al., 1986) and in many cases they and the SK-N-SH parent line have shown a latent ability to morphologically transform either spontaneously or in response to chemical agents such as all-trans-retinoic acid (atRA) from one sub-type to another with concomitant biochemical changes consistent with the altered morphology (Ross et al., 1983). Two types of flat cells can emanate from the SK-N-SH cell line in vitro (Tsokos et al., 1985; Sidell et al., 1986): one with a melanocytic phenotype and one with a Schwann cell phenotype. Cells with a melanocytic phenotype harbour tyrosinase activity (Ross and Beidler, 1983), contain membrane-bound dark structures with lamellar-type striated features characteristic of melanosomes (Tsokos et al., 1987) and express human melanosome-associated glycoprotein antigens (Maeda et al., 1990; Slack et al., 1992). Should structural alterations of the tyrosinase locus be observable, this model may prove valuable as it would facilitate the study of the early trans-acting factors and external variables that are required to activate the locus.

3.4. Repressor Activity of the Human Tyrosinase Coding Region

As the focus of this research was on higher order structural control of tyrosinase gene expression, it was not the intention to delve into transcriptional repression unless it related to the general transcriptional repressive effects experienced by genes bounded by
heterochromatin. However, during the investigation into whether a region in the first exon of the human tyrosinase gene resembling a cAMP (3',5'-cyclic adenosine monophosphate) response element could confer α-MSH (melanocyte stimulating hormone) responsiveness to a transiently transfected luciferase reporter gene, it appeared that the human coding sequences exhibited repressor activity. This serendipitous observation was noteworthy as it might explain a discrepancy of coat pigmentation between transgenic mice generated by Porter and Meyer (1994), whose gene constructs contained human tyrosinase cDNA and transgenic mice established in other studies (Tanaka et al., 1990; Yokoyama et al., 1990) which used mouse tyrosinase cDNA. Hence, it may be beneficial at this juncture to review proposed repression mechanisms, particularly what is known about negative transcriptional control of the tyrosinase gene.

Repression, or negative control, is an important component of gene expression regulation because it provides a mechanism by which gene transcription can be turned off or proactively inhibited. Eukaryotic repressors are involved in ensuring that a gene is transcriptionally silent in those tissues where the gene is not expressed and act quickly to stop the expression of inducible genes once the inducing stimulus is eliminated (Renkawitz, 1990). Repressors are also thought to contribute to the developmental control of gene expression (Dawson et al., 1995; Arnosti et al., 1996). Gene repression is mediated by a variety of proteins that inhibit transcriptional activation by targeting many components of the transcription initiation process (reviewed in Herschbach and Johnson, 1993; Johnson, 1995). Certain repressors prevent activators from being imported into the nucleus by masking vital nuclear targeting sequences on the activator. Alternatively, repressors may compete with activator proteins in the nucleus for the same DNA binding sites or regions close to the
promoter, excluding the activator and preventing transcriptional initiation by that protein. Repressors may bind to an activator that is already bound to the DNA or may take the place of an activator subunit during the assembly of a di- or multimeric activator complex, both functions preventing the activator from interacting with the transcription initiation complex. Inhibitory mechanisms that do not directly involve the basal transcription machinery may provide for more finely tuned suppression as the transcription initiation complex may be able to interact with other activation factors allowing transcription to proceed at a future, perhaps critical period (Renkawitz, 1990).

In addition to the repressor element in the distal enhancer/MAR region of the mouse gene that specifically silences tyrosinase transcription in RPE, negative regulatory elements are found in the promoters of both the mouse and human tyrosinase genes that appear to play a significant, but poorly understood role in transcription modulation. The octamer element that overlaps the Inr E-box in the promoter binds Brn-2/N-Oct3 which may downregulate tyrosinase expression by displacing or preventing the binding of bHLH-Zip proteins, such as Mi, to the Inr E-box motif (Eisen et al., 1995, reviewed in Ferguson and Kidson, 1997). DNase I foot-printing and subsequent deletion analysis of the human tyrosinase promoter points to a protected area between 115 bp and 150 bp upstream of the transcription initiation site as having negative regulatory activity (Bentley et al., 1994) but little is known about this element's role in regulating tyrosinase transcription. In the mouse promoter, the activity of a 68 bp negative regulatory element is able, through sequence mutagenesis, to be abrogated in melanocytes but not in fibroblasts leading to the suggestion that tyrosinase transcription is under negative control and that de-repression is inducible possibly through the displacement of a repressor by a melanocyte-associated external stimulus such as exposure to α-MSH.
The presence of repressor sequences in the coding regions of the rat osteocalcin gene (Frenkel et al., 1993) and human factor VIII gene (Fallaux et al., 1996) attests to the possibility that a repressor element could exist in the human tyrosinase cDNA.

4. Materials and Methods

4.1. General DNase I and Hypersensitivity Assays

Both the general DNase I sensitivity and DNase I hypersensitivity assays are based on the protocol outlined in Porter et al. (1991) which is described here briefly.

4.1.1. Nucleus Isolation

Appropriate cell lines were grown to confluency (see Cells Lines and Culture Methods) on 150 mm culture plates (adherent cells) or in T-75 flasks (suspension cultures). Adherent cell cultures were washed once with 1X PBS, scraped free with a rubber policeman and centrifuged for 5 minutes at 500 x g. Washing was repeated with 10 to 20 mL of 1X PBS and the cells were pelleted once again. Suspension cultures were centrifuged and washed twice with 1X PBS and finally spun down for 5 minutes at 500 x g. Cell pellets were resuspended by vortexing in 1.6 mL RSB-NP40 (10 mM Tris-Cl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40) per 150 mm plate or T-75 flask and the cells were then dounce homogenized until more than 80% of the cells were lysed. The nuclei were separated from cell debris by centrifuging for 10 minutes at 600 x g and the resulting pellet was resuspended by vortexing in 2 mL RSB (without NP-40) per original plate or flask of cells and dounce homogenized a further 8 times.
4.1.2. DNase I Endonuclease Digestion of Genomic DNA

Aliquots of nucleus suspensions (0.5 mL - approximately 6x10^6 cells) were incubated at 37°C for 10 minutes with increasing concentrations (0-10 μg/mL) of DNase I endonuclease. The digestions were stopped by the addition of 0.5 mL of 2X STOP buffer (1% SDS, 600 mM NaCl, 20 mM Tris-Cl (pH 7.5), 10 mM EDTA, 400 μg/mL Proteinase K). The inactivated digests were left overnight at room temperature after which 1 mL of RSB was added to dilute the DNA. The DNA was purified with 2 phenol/chloroform extractions followed by a final chloroform extraction and precipitation with 2.5 volumes of 100% ethanol. The DNA pellets were washed once with 70% ethanol and dissolved in 50 μl of 10 mM:1 mM (10:1) TE buffer (pH 7.5).

4.1.3. Restriction Endonuclease Digestion and Southern Blotting

Ten micrograms of DNA (unless otherwise stated) from each DNase I concentration point were digested using 30 U of the appropriate restriction endonuclease under the conditions recommended by the enzyme manufacturer. The restriction digest was incubated overnight after which 10 U of additional enzyme was added and allowed to incubate at the recommended temperature for an additional 2 hours to ensure the digestion went to completion. Each digestion was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.7) and 2.5 volumes of 100% ethanol followed by a wash with 70% ethanol and the resuspension of DNA in 20 μl 10:1 TE (pH 7.5) buffer. Two microliters of 20% Ficoll loading buffer were added to each of the 20 μl DNA samples which were then horizontally electrophoresed through a 0.8% agarose (Gibco-BRL) gel in 1X TBE (pH 7.0) running buffer for 5 to 16 hours at 2 volts/cm. Upon completion of electrophoresis, the gel was
stained in a 100 μg/mL ethidium bromide solution for 30 minutes, rinsed in ddH₂O and then photographed. Prior to Southern blotting, the gel was washed once for 12 minutes in 0.25 N HCl and twice for 15 minutes each in denaturing solution (1.5 M NaCl, 0.5 M NaOH). The DNA was transferred overnight to Nytran+™ nylon membrane (Schleicher & Schuell) via capillary transfer using 20X SSC (pH 7.0). When transfer was complete the membrane was rinsed with 2X SSC (pH 7.0) buffer, blotted dry, and the DNA was covalently bonded (UV cross-linked) to the membrane by exposing it to 1.2 X 10⁵ joules of 254 nm ultra-violet radiation in a Stratagene Strata-linker™.

4.1.4. Nucleic Acid Probe Synthesis and Hybridization

Southern blots of DNase I digested genomic DNA for DNase I general and hypersensitivity studies were routinely hybridized with α-³²P dCTP random-primed labelled DNA probes. For analysis of hypersensitive sites near the human tyrosinase promoter region an α-³²P UTP labelled riboprobe was utilized.

4.1.4.1. Random-Primed Oligonucleotide Probe Labelling

Twenty five to 50 nanograms of appropriate double-stranded DNA were labelled using a random-primed labelling kit (Pharmacia) following the manufacturer’s directions. The 50 μl reactions, containing 50 μCi of 3000 Ci/mmole α-³²P dCTP (Dupont-NEN) and 9.1 U Klenow enzyme, were incubated at 37°C for 30 to 60 minutes. Unincorporated nucleotides were removed with a Nucleotrap™ column (Stratagene) and the purified probe was denatured at 100°C for 5 minutes and placed on ice prior to hybridization.
4.1.4.2. Riboprobe Synthesis

Human tyrosinase cDNA isolated from BBTY-1 (Bouchard et al., 1989) was used to generate a riboprobe specific for exon 1. Two micrograms of tyrosinase cDNA subcloned into pBlueScript SK II + plasmid vector (Stratagene) were digested to completion with Styl restriction endonuclease (New England Biolabs) in a 25 µl reaction (0.1 volume 10X NEB 3 buffer, 2 mM spermidine, 100 µg/mL BSA, 200 µg/mL RNase A, 6 U Styl) for 1 hour at 37°C. RNases were inactivated following restriction digest by adding 0.1 volume of 10X Proteinase K Buffer (100 mM Tris-Cl (pH 8), 50 mM EDTA (pH 8), 500 mM NaCl), 0.1 volume 5% SDS, and Proteinase K to a final concentration of 100µg/mL and incubating the reaction at 37°C for one hour. The DNA was purified with two phenol/chloroform extractions followed by one chloroform extraction and ethanol precipitation. The DNA was dissolved in 10 µl of 10:1 TE buffer (pH 7.5) and 1 µg was used for riboprobe synthesis in the following reaction: 0.2 volume 5X transcription buffer (200 mM Tris-Cl (pH 8), 40 mM MgCl₂, 10 mM spermidine, 250 mM NaCl, 10 mM each of rGTP, rCTP, and rATP, 10 U RNase inhibitor, 50 µCi 3000 Ci/mmole α-32P UTP (Dupont-NEN), 25 U T7 RNA polymerase, and DEPC (diethyl pyrocarbonate)-ddH2O to 25 µl. The labelling reaction was incubated at 37°C for 30 minutes after which the unincorporated nucleotides were removed from the riboprobe using a Nucleotrap* column (Stratagene). The purified probe was placed on ice prior to hybridization.

4.1.4.3. Probe Hybridization

Prehybridization and hybridization of Southern blots were performed at 42°C for DNA probes and 55°C for riboprobes following Nytran (Schleicher & Schuell) directions.
Prehybridization was performed for 2 hours in 6X SSPE, 10X Denhardt’s reagent, 1% SDS, and 200 mg/mL denatured sonicated herring sperm DNA while hybridization was performed for 12-16 hours in 6X SSPE, 50% formamide, 1% SDS, and 100 mg/mL sonicated herring sperm DNA. Prehybridization and hybridization solutions, 150 μl per cm² of blotting membrane were used. Two 30 minute washes in 2X SSC/0.1% SDS, first at room temperature then at 65°C were performed followed by two 30 minute washes in 0.1X SSC/0.1% SDS at 65°C. The membranes were blotted dry and autoradiographed on Kodak XOMAT-AR film with intensifying screens at -80°C or without screens at room temperature.

For the general DNase I sensitivity probe signals that were to be quantified, the films were pre-flashed with an Amersham Sensitize™ flash unit prior to exposure to ensure signal linearity. The exposed films were then scanned with a hand-held optical densitometer (Advanced American Biotechnology). To determine the rate of signal decrease the intensity of each DNase I concentration point, the signal was scored as a percentage of that for the zero DNase I control.

All routine genomic Southern analyses were performed as described above but without the DNase I digestion. In such cases, the genomic DNA used for such analysis was obtained either from the intact nuclei not digested with DNase I or from tissue culture cells incubated at 55°C with gentle shaking for 12 hours in 50 mM Tris-Cl (pH 8), 50 mM EDTA, 0.5% SDS, 0.5 mg/mL Proteinase K, and 100 μg/mL RNase A.

Using reverse Southern analysis to find non-repetetive sequences in the mtyr1.7NB and mtyro.9NP subclones of the mtyr13 bacteriophage clone representing the upstream regions of the mouse tyrosinase locus (see Figure 2 on page 49), the DNA to be tested was digested with various restriction enzymes and run on a 1% agarose gel to separate the restriction
fragments. The gel was Southern blotted as previously described and then probed with mouse genomic DNA that had been sheared and labelled by the random-primed method described above. Hybridization and probe detection was performed on these blots as described above.

4.2. Northern Blot Analysis

4.2.1. Total Cellular RNA Isolation

Total cellular RNA extraction from either tissue samples or cultured cells was performed on ice with DEPC-treated solutions following the protocol by Chirgwin et al. (1979). Tissue/cells were dounce homogenized in Solution D (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% Sarkosyl, and 0.7% β-mercaptoethanol) then vigourously mixed with 0.1 volume of 2 M sodium acetate (pH 4.7), 1 volume of ddH$_2$O, 0.2 volume of chloroform:isoamyl alcohol (23:1) and kept on ice for 15 minutes after which the mixture was centrifuged at 10,000 x g at 4°C for 20 minutes. The RNA was precipitated from the supernatant with ice-cold isopropanol at -20°C for 1 hour, dissolved in 0.5 mL of Solution D and then precipitated again. The RNA pellet was washed twice with ice-cold 75% ethanol and dissolved in DEPC-10:1 TE (pH 7.5).

4.2.2. Gel Electrophoresis and Northern Blotting

Ten micrograms (unless otherwise stated) of each RNA sample was prepared in a 40 μl volume with the following reagents at the final concentration specified: 50% formamide, 17.5% of 37%(v/v) formalin, and 1X MOPS buffer. The samples were heated to 65°C for 15 minutes, placed on ice for 5 minutes, then run on a 1% agarose/formaldehyde gel (18%
of 37%(v/v) formalin solution, 1X MOPS buffer) in 1X MOPS running buffer at 3 to 5 volts/cm for 3 hours. Upon completion of electrophoresis, the gels were stained with 100 μg/mL ethidium bromide for 20 minutes, destained with DEPC-ddH₂O to remove background staining, then photographed. The gels were blotted as previously described for Southern blotting but without depurination and denaturation and using DEPC-treated 20X SSC (pH 7.0) transfer buffer. Nucleic acid labelling and probe hybridization procedures were performed as described above.

4.3. Cell Lines and Culture Methods

All growth media and supplements as well as antibiotics and antifungal agents were purchased from Gibco/BRL unless otherwise stated. All cell lines were cultured at 37°C in 5% CO₂ in air.

Cloudman S91 (clone M-3) Mouse Melanoma

This cell line was obtained from the American Type Culture Collection (ATCC) (cat. # CCL 53.1) and cultured in Ham's F-10 medium supplemented with 18% horse serum, 3% foetal calf serum, 25 IU penicillin/mL, and 25 μg streptomycin/mL. The culture medium was changed every three days.

SK-Mel-28 Human Melanoma

This cell line was obtained from the ATCC (cat. # HTB 72) and maintained in Eagle MEM with Hanks' BBS and non-essential amino acids supplemented with 1 mM sodium pyruvate, 10% foetal calf serum, 25 IU penicillin/mL, and 25 μg streptomycin/mL. The culture medium was changed every three days.
SK-N-SH Human Neuroblastoma

Obtained from the ATCC (cat. # HTB-11), this cell line was cultured in Eagle MEM with Earle’s BBS supplemented with 100 \( \mu \)M non-essential amino acids, 1 mM sodium pyruvate, 10% foetal calf serum, 25 IU penicillin/mL, and 25 \( \mu \)g streptomycin/mL. The culture medium was changed every three days.

SK-N-SHF Human Neuroblastoma

This cell line is an ‘epithelial-like’ subclone derived from the SK-N-SH human neuroblastoma cell line and was a gift from Dr. Neil Sidell (Department of Pathology, UCLA School of Medicine, Los Angeles, CA). It was maintained in 1640 RPMI supplemented with 10% foetal calf serum, 25 IU penicillin/mL, and 25 \( \mu \)g streptomycin/mL. The culture medium was changed every three days. For differentiation studies, \( 1 \times 10^4 \) SK-N-SHF cells (per culture dish) were inoculated onto 90 mm culture dishes with 10 mL of culture medium and grown to 30 to 40% confluence at which point all-trans-retinoic acid (ataRA) was added to the medium to a final concentration of \( 3 \times 10^{-6} \) M. The ataRA was added at this concentration when the medium was changed on every third day. The ataRA was initially dissolved in absolute ethanol so an ethanol vehicle control was also set up where the SK-N-SHF cells were incubated in the presence of 0.024\%(v/v) ethanol (the same as the ataRA-treated cells).

HeLa Human Cervical Epithelioid Carcinoma

This cell line was a gift from the Dr. D.E. Brooks’ laboratory (Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC) and was cultured in Eagle MEM with Earle’s BBS and non-essential amino acids supplemented
with 10% foetal calf serum, 25 IU penicillin/mL, and 25 μg streptomycin/mL. The culture medium was changed every third day.

**U937 Human Histiocytic Lymphoma**

Obtained from ATCC (cat. # CRL 1593), this cell line, originally submitted to ATCC by Sundstrom and Nilsson (1976) was maintained in 1640 RPMI supplemented with 10% foetal calf serum, 25 IU penicillin/mL, and 25 μg streptomycin/mL. The culture medium was changed every three days.

### 4.4. Genomic Library Screening and Construction

#### 4.4.1. Mouse Genomic DNA Library Screening

Two mouse genomic DNA libraries were screened: a bacteriophage genomic library and a cosmid genomic library. Both the bacteriophage library (Stratagene; cat. # 946305) and the cosmid library (Stratagene; cat. # 961301) were derived from 129SVJ female mouse liver DNA partially digested with *Sau3AI* restriction endonuclease and inserted into either the λ-Fix II bacteriophage vector or the Cosmid SuperCos 1 vector (Stratagene). Screening of these libraries was performed as recommended by Stratagene and is briefly outlined here.

For the bacteriophage library, recombinant clones were transfected into *E. coli* LE392 host bacteria, mixed with 0.7% LB (Luria-Bertani)-top agarose, and overlaid onto 1.5% LB-agar at a density of 5x10⁴ plaque-forming units (pfu) per 150 mm Petri plate. Recombinant clones of the cosmid library were inoculated at a density of 5x10⁴ colony-forming units (cfu) per 150 mm petri plate onto LB-agar containing 100 μg L-ampicillin/mL. After overnight growth at 37°C in ambient air, the nearly confluent plaques or colonies were transferred to BA-S nitrocellulose membranes (Schleicher & Schuell), the DNA was denatured and the blots
neutralized. The membranes were blotted to dryness and the DNA was UV cross-linked. The membranes were hybridized as previously described, washed under stringent conditions and autoradiographed. The autoradiograms were positionally matched with the appropriate petri plates and any positive pfu or cfu were subsequently picked off the plates and isolated as above. A total of approximately $2 \times 10^6$ pfu from the bacteriophage library were screened in two separate screenings, each time with a different radiolabelled DNA probe (see Figure 2A on page 50): the first screening was probed with $mtyr0.65X$ (probe 5) and the second screening was probed with $mtyr0.9EX$ (probe 6) the later probe being immediately upstream of $mtyr0.65X$. A total of $2.25 \times 10^6$ cfu from the cosmid library were screened, also in two separate screenings, using $mtyr0.7E$ (probe 3) for both.

4.4.2. Human Genomic DNA Library Screening

Two pre-made human genomic bacteriophage libraries were screened in a manner similar to that just described. A leukocytic genomic DNA library, partially digested with MboI and cloned into the λMG14 vector (Rotwein, 1986 a,b) was obtained from the American Type Culture Collection (ATCC; cat. # 37458). Transfecting the recombinants into C600 E. coli bacterial host cells, a total of $1 \times 10^6$ pfu were screened with $htyr0.7EX$ (probe d, see Figure 5A on page 62). A placental genomic library (Clonetech; cat. # HL 1067j) partially digested with Sau3AI and inserted into the BamHI site of the EMBL3 SP6/T7 vector was transfected into the K802 E. coli host bacterial strain and a total of $2.6 \times 10^6$ recombinants were screened with $htyr0.7EX$ (probe d).
4.4.3. Construction of SK-Mel-28 Human Melanoma Sub-Genomic Library

A sub-genomic library was constructed using SK-Mel-28 DNA which is shown by this study to contain the -10.5 kb DHS within a 15 kb BgII restriction fragment. DNA was isolated from SK-Mel-28 cells and purified as described above and digested with BgII restriction endonuclease until digestion was complete. The DNA was subsequently size fractionated using a 0.8% agarose gel in 1X TBE buffer and electrophoresis for 18 hours at 2 volts/cm. Measured from the top of the gel, 0.5 cm gel slices were excised and the DNA from each fraction was purified using Qiaex II DNA purification system (Qiagen). The DNA from each 0.5 cm fraction was electrophoresed to determine the degree of shearing and to confirm which fraction contained the 15 kb DNA fragments. One lane of the original agarose gel used to size fractionate the DNA was Southern blotted and probed as previously described with htyr0.7EX DNA (probe d). A hybridization signal (data not shown) was detected 2.2 cm from the top of the lane correlating with the 15 kb region being represented by the purified DNA fraction isolated from the 2 to 2.5 cm region measured from the top of the gel. Following Stratagene protocols, the BgII ends of the purified DNA fragments from the 2 to 2.5 cm fraction were partially filled-in with dATP/dGTP nucleotides in the presence of Klenow enzyme to render the ends compatible with the XhoI/partial fill-in ends of the λ-FIX II bacteriophage vector arms (Stratagene). To control for the restriction digests, the partial fill-in, and the subsequent ligation, two different plasmids were taken through each step simultaneously and in the same manner as the test DNA: pBluescript (pBS) II SK+ phagemid DNA (Stratagene) was digested with XhoI, partially filled-in with dTTP/dCTP (emulating what was done to the vector arms by Stratagene) and ligated to pLitmus29 plasmid DNA (New England Biolabs) that was digested with BgII and partially filled-in with
dATP/dGTP (emulating what was done to the SK-Mel-28 DNA). The two plasmids were ligated together in the same DNA to reaction volume ratio as the test DNA. An aliquot of each control digest before and after partial fill-in was self-ligated and gel electrophoresed along with an aliquot of the PBS II SK+/pLitmus29 ligation to determine that each step proceeded correctly. The genomic DNA fragments were ligated to the bacteriophage arms and packaged using Gigapack III Gold (Stratagene) packaging extract following the manufacturer's directions. To ensure that the library contained viable clones, a titre was performed on the stock library using XL1-MRE (P2) strain of *E. coli* host cells. The stock library then underwent one round of amplification to increase and stabilize the titre. The amplified sub-genomic library was screened with *htyr0.7EX (probe d)* as described above.

4.5. DNA Sequencing

All DNA sequenced was subcloned into the pBluescript II SK+ phagemid vector (Stratagene) using standard subcloning methods (Ausubel *et al.*, 1994) and transformed into DH5α library efficiency competent cells (Gibco/BRL). Recombinant plasmids were isolated by alkaline miniprep technique (Ausubel, 1994) followed by phenol/chloroform extraction and alcohol precipitation purification. The sequencing reactions were performed using the Sequenase (version 2.0) sequencing kit (United States Biochemical), 5μCi 800 Ci/mmol α-S35 dATP (Dupont-NEN), and a variety of primers (T7, T3, KS, or SK) following the sequencing kit manufacturer's protocol. The sequencing reactions were electrophoresed in a 6% polyacrylamide denaturing gel (8M urea) in 1X TBE (pH 7.0) running buffer at 1600V. Electrophoresed gels were transferred to Whatman 3M paper, dried, and exposed on Kodak XOMAT AR autoradiograph film over night at room temperature.
4.6. Polymerase Chain Reaction (PCR) Amplification

4.6.1. Amplification of the htyr0.18US (probe c)

The oligonucleotides a) 5’GGGGGTACCGAGTACAACGTGTAGGCC3’ and b) 5’GGGGAGCTCGGGCTTTTGTGTCTGCTGTC 3’ with KpnI and SacI 5’ ends, respectively, to facilitate subcloning, were used to prime the amplification of a 182 bp fragment (htyr0.18US) representing the region immediately 5’ of htyr1.5E from SK-Mel-28 genomic DNA. Each 25 μl reaction, containing 0.2 mM dNTPs (Pharmacia), 1.5 mM MgCl₂, 25 mM each primer, and 0.1U Taq polymerase (Promega), was amplified for 30 cycles: each cycle consisting of 1 minute each at 94°C, 52°C, and 72°C. The amplification reactions were electrophoresed on a 2% agarose gel and the 182 bp fragments were excised and purified using Qiaex (Qiagen) and then directionally subcloned into the pBluescript II SK+ phagemid vector (Stratagene) using standard sub-cloning procedures as found in Ausubel et al. (1994). Recombinant htyr0.18US was confirmed by sequencing as described above.

4.6.2. Amplification of -10.5 kb Hypersensitive Region from SK-Mel-28 genomic DNA

SK-Mel-28 genomic DNA was digested with NcoI, precipitated and redissolved in 10:1 TE buffer (pH 7.5). The fragments were ligated on themselves with T4 DNA ligase and then the circular DNA was linearized by cutting with Nsil. Two sets of oligonucleotide primers were used for the inverse PCR; both sets were designed to anneal at the 5’ and 3’ ends of htyr0.7XE on either side of the Nsil site 250 bp in from the 5’ end of htyr0.7XE, in the orientation illustrated in Figure 5: set a - 5’CACACCAAATGCCTAGAGAAACATTTC3’ and 5’CCTGAGATGTATCTTACAGTATGTGTGAG3’; set b (nested) -5’GGAGATGGGAG
AATGGATGGATGAGTG3' and 5'GGTTGCTAACATGAGAAGGAGAGACC3'. The controls included: 3.7 kb \textit{htyr0.7EX} subcloned into pBluescript II SK+ (\textit{htyr0.7EX/pBS II SK+}; both linear and circular), 4.5 kb \textit{htyr4.5E} ligated on itself, and 7.5 kb \textit{htyr4.5E} subcloned into pBluescript II SK+ (\textit{htyr4.5E/pBS II SK+}). \textit{Taq-Plus} (Stratagene) was used as the DNA polymerase for its ability to generate PCR amplification products of up to 35 kb in length.

4.7.\hspace{1em} Transient Transfection Analysis

4.7.1. \textit{Luciferase} Reporter Gene Construction

Two \textit{luciferase} reporter plasmid vectors were used to make the recombinant reporter gene constructs containing human \textit{tyrosinase} cDNA or parts thereof: the \textit{pGL2-promoter} vector (\textit{pGL2pro}, Promega) driven by the heterologous SV40 early promoter and the \textit{pGL2-basic} vector (\textit{pluc}, Promega) driven by 2.5 kb of mouse \textit{tyrosinase} promoter region inserted into the \textit{SmaI/BglII} cloning sites upstream of the \textit{luciferase} coding sequences. The human \textit{tyrosinase} cDNA sequences were subcloned in a 3' to 5' orientation into the unique \textit{BamHI/SalI} restriction sites downstream of the \textit{luciferase} coding sequences. The following is a brief outline of how each of the constructs used was made (see Figure 9A on page 86). For each construct, the plasmid vectors and human \textit{tyrosinase} cDNA that were cut with restriction enzymes during preparation were electrophoresed in 1% agarose gels to separate the resulting fragments. The fragments of interest were then excised from the gels, purified using Qiaex II (Qiagen) and subsequently dissolved in 10:1 TE buffer (pH 7.5). All constructs synthesized were confirmed by restriction digest analysis.
These constructs contained the entire 1888 bp human tyrosinase cDNA. The cDNA was excised from pBS II SK+ with BamHI and SalI and, using standard subcloning protocols (Ausubel et al., 1994), ligated with the respective plasmid vector DNA also cut with BamHI and SalI. The human tyrosinase cDNA was originally subcloned into the EcoRI site in pBS II SK+ so when released with BamHI and SalI, 19-27 bp of pBS II SK+ sequence from the multiple cloning site (MCS) remained on the ends of the cDNA insert.

These constructs contained exon 1 and 126 bp of exon 2 from the human tyrosinase cDNA. The pGL2 and pluc vectors were cut with BamHI, blunt-ended using Klenow (Pharmacia) and dNTP’s (Pharmacia) following manufacturer’s directions, and then cut with SalI. The cDNA was cut with SalI and PvuII and the 952 bp fragment containing exon 1 and part of exon 2 was subcloned into the respective reporter plasmid vectors. The cDNA insert was flanked at the 5’ end by 27 bp of MCS between the SalI site and the EcoRI site originally used to subclone the human tyrosinase cDNA.

These constructs contained the remaining 94 bp of exon 2 and exons 3, 4, and 5 from the human tyrosinase cDNA. The pGL2 and pluc vectors were cut with SalI, blunt-ended, and then cut with BamHI. The cDNA was cut with BamHI and PvuII and the 936 bp fragment containing the remainder of exon 2 and exons 3, 4, and 5 was subcloned into the respective reporter plasmid vectors. The cDNA insert was flanked at its 3’ end by 19 bp of MCS between the BamHI site and the EcoRI site originally used to subclone the human tyrosinase cDNA.
This construct contained the first 59 bp of human tyrosinase exon 1 and was made from pGL2-3'exl. pGL2-3'exl was digested with BamHI and BclI which cuts 59 bp downstream of the 5' end of exon 1. The BamHI end was blunt-ended and the construct was religated on itself.

4.7.2. Cell Line Transfection

Prior to transfection, DNA constructs and plasmid reporter vector controls were purified by extracting the DNA twice with phenol:chloroform:isoamyl alcohol followed by one extraction with chloroform alone. The constructs were ethanol precipitated and dissolved in ddH₂O. Transient transfection of the various cell lines was done using the directions provided with the LIPOFECTIN™ reagent (Gibco/BRL) as a guide and is outlined here briefly. The following cell lines were used for the transient transfection assays: S91 mouse melanoma, SK-Mel-28 human melanoma, and HeLa human cervical epitheloid carcinoma. Approximately 3.8x10⁵ cells of each cell line were inoculated to 35 mm culture dishes and cultured in the appropriate media (see Cell Lines and Culture Methods) until 30% to 40% confluent growth had been achieved. At that time (approximately 24 hours), the cells were rinsed once with 1 mL of 1X PBS followed by twice with 2 mL each time of the appropriate culture media without serum or penicillin/streptomycin. Each of the DNA constructs and reporter plasmid vector controls were co-transfected with an equimolar amount of pSV-β-galactosidase reporter plasmid (Promega) to control for transfection efficiency differences. A control containing only the reagents and no DNA was included for each transfection. For each DNA construct and control the transfections were performed in triplicate. The total
amount of DNA and the volume of LIPOFECTIN in each transfection was held consistent at a 1:7 ratio (µg DNA:µl LIPOFECTIN). The DNA and LIPOFECTIN were each mixed separately with 100 µl of the appropriate serum-free medium and then combined and incubated at room temperature for 15 minutes. An additional 800 µl of the appropriate serum-free medium was added to the DNA:LIPOFECTIN mixture which was then then overlaid on the cells in each culture dish. The cells were incubated overnight and on the following day, the medium in each culture dish was replaced by the appropriate medium containing serum and penicillin/streptomycin. The cells were incubated an additional 2 days until they were almost confluent and then cell lysates were prepared by washing the cells once with 2 mL of 1X PBS and then incubating the cells in 150 µl of 1X RLB (reporter lysis buffer, Promega) at room temperature for 15 minutes after which the cells were scraped off the dish with a rubber policeman into a microcentrifuge tube. The lysates were centrifuged at 13,000 x g to pellet the cells and debris and the supernatents were aspirated off and, unless analysed immediately, were stored at -70°C until luciferase and β-galactosidase activity assays could be performed.

4.7.3. Reporter Gene Activity Determination

To ensure the sample volumes used in each assay resulted in values that were within the linear detection range, the linear ranges of the Luciferase Assay System (Promega) and the Galacto-Light Plus™ (Tropix) β-galactosidase assay kit were determined by assaying progressively larger volumes of pGL2-3'htyr S91 cell lysate for the respective activities. Prior to determining the β-galactosidase activity, the endogenous β-galactosidase was inactivated by heating the cell extract sample to 48°C for 50 minutes. The assays were
performed following the kit manufacturer's directions using a liquid scintillation counter to monitor each reaction's chemiluminescent signal. After subtracting the background luciferase and β-galactosidase values for the reagents only (no cell lysate), the average luciferase/β-galactosidase values for each construct of each transfected cell line were plotted (see Figure 9B on page 86) on a bar graph as a percentage of the average luciferase/β-galactosidase value of the nonrecombinant (i.e., promoter only) pGL2 or pluc vectors. The error bars on both Figure 9B graphs represent the mean of the three relative CPM values for each construct ±SEM.

5. Is the Mouse Tyrosinase Enhancer/MAR the 5' Domain Boundary?

5.1. Results

The general DNase I sensitivity was determined for the mouse tyrosinase coding and upstream regions flanking the enhancer/MAR of cultured S-91 mouse melanoma cells and compared to the sensitivity of bulk chromatin represented by a non-expressed gene. Through screening mouse genomic DNA bacteriophage and cosmid libraries, there was an attempt to isolate non-repetitive DNA sequences upstream of the MAR that could be used as probes to determine the point at which the DNase I sensitivity of the 5' regions flanking the MAR becomes comparable to bulk chromatin. Such a transition of chromatin structure relatively close to the MAR would implicate the MAR as the 5' boundary of the mouse tyrosinase locus.

5.1.1. General DNase I Sensitivity of the Mouse Tyrosinase Locus

To determine nuclease sensitivity of the upstream regions of the mouse tyrosinase gene
locus and bulk genomic DNA, tyrosinase-positive S91 mouse melanoma cell nuclei were isolated and treated with increasing concentrations of DNase I endonuclease. Following the DNase I digestion, the nuclei were lysed and the genomic DNA from each concentration point was purified and cut with either XbaI or HindIII. The DNA restriction digests were separated by electrophoresis, Southern blotted and hybridized with DNA probes representing either mouse tyrosinase coding sequences, the sequences flanking either side of the upstream enhancer/MAR or coding sequences of the Cμ immunoglobulin gene which is not expressed by melanocytes (see Figure 2A). The HindIII Southern blots were hybridized with probe 1, a 3.3 kb mouse tyrosinase cDNA probe (Terao et al., 1989). The XbaI Southern blot was initially hybridized with probe 2 (mtyr1H), a 1 kb HindIII fragment downstream of the 5' enhancer/MAR, then stripped and simultaneously hybridized with probe 3 (mtyr0.7E), a 0.7 kb EcoRI fragment immediately upstream of the enhancer/MAR and probe 4, a 1 kb BamHI/XbaI fragment of Cμ cDNA containing the Cμ first exon (see Kawakami et al., 1980).

The method that was used to assess the degree of open conformation of the various regions was the ‘first cut’ method used by others to examine the chromatin structure of the chicken β-globin gene (Wood and Felsenfeld, 1982), define the 5' and 3' boundaries of the chicken lysozyme gene (Jantzen et al., 1986), and to characterize the chinchilla-mottled mouse mutation as one that alters the chromatin structure of the mouse tyrosinase coding region (Porter et al., 1991). This method, also known as a fade-out assay, measures the degree of DNase I sensitivity of a region of DNA as the concentration of DNase I increases. The degree of sensitivity is demonstrated by a progressive decrease of signal intensity, as the DNase I concentration increases, of a restriction band representing the region of interest on
Figure 2. Map of Upstream Regions and DNase I Hypersensitive Sites of the Mouse Tyrosinase Locus.

A. The middle map represents upstream regions of the mouse tyrosinase locus showing the positional relationship between the promoter and -15 kb enhancer/MAR DNase I hypersensitive sites (vertical arrows) and the inverted L1Md element upstream of the MAR. The enlarged map at the top shows detail of the MAR and associated flanking regions as well as the DNA probes (solid rectangles) and the restriction fragments they hybridize to in the general DNase I sensitivity assays. Probe 1 (mouse tyrosinase (mtyr) cDNA) binds to the 4.8 kb (exon 1) and 2.2 kb (exon 5) HindlU fragments (as well as exons 2, 3, and 4 - not shown); probe 2 (mtyr1H) binds to a 4.4 kb Xbal fragment; probe 3 (mtyr0.7E) binds to a 1.9 kb Xbal fragment. Probe 3, probe 5, (mtyr0.65X) and probe 6 (mtyr0.9EX) were used to screen the mouse genomic DNA libraries. The dashed lines at either end of the L1Md element denote the uncertainty of the exact positions of the 3' and 5' LINE termini. A, Apal; B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; N, NotI; P, PstI. The brackets around the NotI sites indicate that these sites are subcloning artifacts and are not present in the mouse genome.

B. The mtyr13 bacteriophage clone from the 129SVJ mouse genomic DNA library is shown from which the 8 kb upstream NotI/Apal fragment, mtyr5'NA8, was subcloned. The origin of the subclones mtyr0.9NP and mtyr1.7NB is shown on the bottom left. The solid arrows represent the sequenced ends of mtyr0.9NP. The map of mtyr5'NA8 shows an internal structure similar to that of a L1Md characterized by Burton et al. (1986). The asterisk (*) on the 3' 2.2 kb BgII fragment indicates that this restriction fragment is longer than the 1.85 kb BgII fragment usually found at the 3' end of known L1Md elements.
the autoradiogram. The lower the DNase I concentration at which the signal intensity diminishes to 50% of the initial restriction fragment intensity (no DNase I), the more sensitive, hence, more open that chromatin region is deemed to be. Figure 3 shows that as one travels upstream from the mouse tyrosinase coding regions to the region immediately 5' of the enhancer/MAR there is a progressive decrease of sensitivity to DNase I. The graph clearly illustrates that the restriction bands found in the tyrosinase coding region (4.8 kb and 2.2 kb HindIII fragments - probe 1) are 2-3 times more sensitive to the effects of DNase I than the restriction fragment representing the region immediately 3' of the enhancer/MAR (4.4 kb XbaI fragment - probe 2), 5 times more sensitive than the region immediately 5' of the enhancer/MAR (1.9 kb XbaI fragment - probe 3), and 7 times more sensitive than a region of DNA representing the coding region of the non-expressed Cμ gene (2.4 kb XbaI fragment). The general DNase I sensitivity of the tyrosinase coding regions relative to bulk (non-expressing) DNA approaches the expected one order of magnitude difference (see Gross and Garrard, 1988).

The purpose of assessing the DNase I sensitivity of the chromatin around the 5' enhancer/MAR was to characterize this region as the 5' domain boundary of the mouse tyrosinase locus. If this hypothesis is true then one might expect the chromatin on the 5' side of the enhancer/MAR to be less sensitive to DNase I digestion than the chromatin on the 3' side and to demonstrate a sensitivity comparable to that of bulk chromatin. Since the sensitivity progressively decreased as the distance upstream from the coding region increased, the enhancer/MAR may be at or very near the end of the domain. However, this cannot be determined with certainty until a DNA region is reached that shows sensitivity equal to that of bulk DNA. When hybridized with probe 3, the region immediately 5' of and
Figure 3. General DNase I Sensitivity of the Coding and Upstream Regions of the Mouse Tyrosinase Locus.

A. Fade-out assays of S91 mouse melanoma DNase I-treated genomic DNA digested with either HindIII or XbaI and hybridized with probes representing mouse tyrosinase cDNA, Cμ coding sequences or the regions flanking the mouse tyrosinase upstream MAR. The autoradiograms show the loss of hybridization signal due to restriction fragment degradation as the DNase I concentration increases from the zero DNase I control lane (gray arrowhead). The size of each fragment (in kb) is noted at the right of each autoradiogram. See Figure 2 for the mouse tyrosinase (mtyr) probe designation and location. The symbols associated with each autoradiogram are used in Figure 3B as markers to denote the corresponding DNase I concentration points.

B. Graphical representation of the DNase I fade-out assays plotting the % hybridization signal (relative to the zero DNase control) as a function of the DNase I concentration.
encompassing some of the MAR displayed about 1.4 times greater DNase I sensitivity than
the non-expressed \( C\mu \) coding region, representing bulk DNA, hybridized with the \( C\mu \) cDNA
probe. Therefore, an attempt was made to isolate DNA further upstream of the
enhancer/MAR to use as a suitable probe for DNase I sensitivity assays of the chromatin
upstream of the MAR.

5.1.2. Searching for Non-Repetitive DNA Sequences Upstream of the Enhancer/MAR

Approximately 4 kb of upstream DNA adjacent to the MAR had been previously isolated
by Ruppert et al. (1988) and was known to contain repetitive LINE (long interspersed
nucleotide repeat element) sequences belonging to the L1 (long interspersed sequence one)
family, oriented in a 3' to 5' direction (see Figure 2A). Southern blot analysis of mouse
genomic DNA (data not shown) using probes isolated from the region immediately upstream
of mtyr0.7E (probe 3) revealed that the 5' end of the LINE element terminated within the 850
bp XbaI/EcoRI fragment. This required that a mouse genomic library be screened to obtain
clones containing DNA sequences sufficiently upstream of the repetitive elements that could
be used as probes. A 129SVJ mouse genomic DNA bacteriophage library was screened
twice, as described in the Materials and Methods, yielding 16 positive clones, one of which
contained 8 kb of DNA upstream of the MAR, the longest stretch of upstream sequences of
all the 16 library clones. This 17 kb clone, mtyr13 (Figure 2B), was purified and digested
simultaneously with \( NolI \) and \( ApaI \) to liberate the upstream region which was subsequently
subcloned (mtyr5'NA8) to facilitate restriction mapping. The 4 kb region between the
\( HindIII \) and \( ApaI \) sites of mtyr5'NA8 had previously been mapped in the laboratory but the
remaining upstream regions had not. Once the upstream region was mapped, two fragments
from the 5' end of mtyr5'NA8 were subcloned to facilitate the search for non-repetitive sequences: mtyr0.9NP, a 0.9 kb NotI/PstI fragment and mtyr1.7NB, a 1.7 kb NotI/BamHI fragment.

Because L1 elements tend to be 7 kb or less in length (Burton et al., 1986) a reasonable place to begin the search for non-repetitive DNA was within the 1.7 kb NotI/BamHI region at the 5' end of mtyr5'NA8. This is where a 7 kb LINE could terminate given that its 5' end starts just upstream of the MAR. To determine which regions contained repetitive sequences, restriction fragments of mtyr1.7NB (data not shown) were analysed by reverse Southern analysis. Fragments that did not detectably hybridize with the labelled mouse genomic DNA were purified, labelled and used to probe a Southern blot of mouse genomic DNA, the latter method being a more sensitive measure of the presence of repetitive sequences. All mtyr1.7NB sub-fragments that were not detected with the labelled genomic DNA probes were subsequently found to hybridize to essentially all regions of mouse genomic DNA, as demonstrated by the resulting smeared pattern on the autoradiograms (not shown), indicating that the probe fragments contained repetitive sequences. A similar analysis was performed on mtyr0.9NP, except the ends were first sequenced in order to find sites for candidate restriction enzymes. No fragments were seen that did not contain repetitive sequences. A mouse cosmid library was also screened twice using probe 3 but no positive clones were isolated.

The mtyr0.9NP sequences obtained were compared to known DNA sequences using PCGENE (version 2) software and the CDEM37RM database therein. From the 3' (PstI) end, 325 bp showed 58% homology with a rat LINE linking the vasopressin and oxytocin genes (Schmitz et al., 1991). The 99 bp of the 5' (NotI) end demonstrated 61% homology
with the L1Md-A13 repetitive element (Shehee et al., 1987) and 54% homology with another L1Md element (Loeb et al., 1986), both of which are found in the BALB/c (albino) mouse genome.

5.2. Discussion

5.2.1. DNase I Sensitivity Decreases Around the Upstream MAR

The general DNase I sensitivity of the tyrosinase locus progressively decreased from the coding regions out towards the upstream MAR. Contrary to what one might expect if the MAR was the 5' tyrosinase domain boundary, the chromatin architecture outside the MAR does not appear to change abruptly immediately upstream of the MAR to a level that is comparable to that of bulk DNA. A gradual decrease of the chromatin sensitivity has been observed at the 5' MAR boundary elements of other gene domains (Lawson et al., 1982; Alevy et al., 1984; Jantzen et al., 1986) such that the sensitivity is not equivalent to that of bulk DNA for a considerable distance from the MAR. It is possible that this effect is a result of subtle differences from cell to cell and is seen when the sensitivity of millions of cells is assayed simultaneously (Lawson et al., 1982). It may be that chromatin condensation occurs around the MAR at slightly different locations in each cell due to the imprecise redistribution of histone H1 around the MARs that is associated with open chromatin (Zhao et al., 1993) or to the variable extents of torsional stress at the MAR (Jantzen et al., 1986).

In the transcriptionally active mouse tyrosinase gene, both the coding and the 5' flanking regions were more sensitive to DNase I digestion than was bulk DNA represented by the coding region of the non-expressed Cμ gene. Although one fragment representing the mouse tyrosinase coding sequences would have sufficed to show that the transcriptional region is
most sensitive, both the first and fifth exon fragments were included in the sensitivity analysis to show that the difference of DNase I sensitivity between the restriction fragments in this assay was influenced minimally by fragment size. The entire coding region of a gene tends to be equally sensitive throughout (see Jantzen et al., 1986) and it appears that this is indeed the case as both exon 1 and exon 5 fragments were 50% degraded at around 1mg/mL of DNase I, although the exon 5 fragment appears to be slightly more sensitive than the exon 1 fragment. Given the higher number of potential cleavage sites for the DNase I enzyme on a larger DNA molecule, one might expect that the larger 4.8 kb HindIII fragment containing exon 1 would have exhibited heightened sensitivity to the effects of lower concentrations of DNase I than the 2.2 kb HindIII fragment harbouring exon 5 but it is evident that this is not the case. Thus, assuming that the chromatin structure of the coding region is uniform, it would appear that within the size range of the fragments assayed the degree of sensitivity depends predominantly on chromatin structure and that the slight sensitivity difference between the two fragments may be due to inherent random errors associated with the method.

The first exon fragment also harbours part of the promoter region; the hypersensitive site is located very close to the 5' end of the 4.8 kb HindIII fragment upstream of the tyrosinase cDNA probe recognition sequences. As the presence of DNase I hypersensitive sites within the fragment being assayed can increase the degree of general DNase I sensitivity (Jantzen et al., 1986; Dillon and Grosveld, 1994) one might expect that this also would result in the larger exon 1 fragment being more sensitive, but the influence, if any, of the hypersensitive site on the DNase I sensitivity of the 4.8 kb HindIII fragment appears to have been minimal.
5.2.2. An Upstream LINE Impedes General DNase I Sensitivity Determination

Probe 3 was the most 5' non-repetitive mouse *tyrosinase* sequence readily available. However, when used to measure the general DNase I sensitivity upstream of the MAR it did not provide unequivocal evidence that the MAR was the 5' boundary of the *tyrosinase* locus. It was imperative that non-repetitive DNA probe sequences be found upstream of MAR so that it could be determined whether or not the region upstream of the MAR was outside of the *tyrosinase* locus, adopting the condensed structure of bulk chromatin. This search was hindered by an approximately 7 kb LINE adjacent to the enhancer/MAR and possibly a second, immediately flanking LINE. LINEs are highly repetitive transposable elements that are found throughout mammalian orders and are present in upwards of 100,000 copies per genome (Burton *et al.*, 1986; Casavant and Hardies, 1994). Their function is unknown although members of the L1 family consist of pseudogenes containing both long and short open reading frames signifying that they express proteins or at least have done so in the past (Loeb *et al.*, 1986; Burton *et al.*, 1986). The LINE upstream of the MAR displays a remarkable structural similarity to the approximately 6.4 kb L1Md (Md: *Muscularis domesticus*) element found 3' of the mouse β-globin gene (Burton *et al.*, 1986). The marked restriction fragment lengths on the *mtyr5'NA8* map are similar to those restriction fragment lengths of the β-globin L1Md except for the most 3' 2.2 kb *BglII* fragment which is 1.85 kb in the consensus L1Md sequence of Burton *et al.* (1986). The 3' terminus of the L1Md element mapped by Burton *et al.* occurs about 250 bp downstream of the 1.85 kb *BglII* fragment and is marked by a poly(A) sequence. L1Md elements share a common 3' end and the variable length of L1Md elements is due to random truncations (Burton *et al.*, 1986; Loeb *et al.*, 1986; Shehee *et al.*, 1987) and/or *BamHI* polymorphisms at their 5' ends.
(Burton et al., 1986). Thus, it would seem reasonable to predict that the 3' end of the LINE would reside in mtyr1.7NB, possibly in mtyr0.9NP. The results of both types of Southern blot analysis and the considerable nucleotide sequence homology with other LINEs demonstrated by mtyr0.9NP would indicate that the 5' end of mtyr5'NA8 is indeed repetitive. However, whether it represents the 3', perhaps polymorphic, end of an unusually long (>7 kb) L1Md element or a separate, juxtapositioned LINE remains to be determined.

With the large number of bacteriophage clones that were detected using probe 5 and probe 6, initially it appeared promising that DNA sequences sufficiently upstream of the MAR and flanking repetitive elements could be isolated from the bacteriophage libraries. This not being the case, the 129SVJ genomic DNA library prepared with a cosmid cloning vector was screened with the expectation that at least one of the larger DNA inserts would span the distance covering the repetitive elements between the MAR and the non-repetitive upstream sequences. Each step of the cosmid library screening procedure was controlled for by simultaneous processing of a nitrocellulose blot bound with mtyr13 bacteriophage plaques. The control blot hybridized successfully with probe 3, indicating that the screening procedure was performed correctly. Thus, it is likely that the inability to detect any cosmid library clones harbouring the mtyr0.7E sequence was due to their absence from the screening blots. Additional screenings of the cosmid library might result in the detection of positive clones. Alternatively, screening a chromosome 7-specific DNA library or a library prepared from a YAC containing the tyrosinase gene would increase the relative number of clones containing DNA from the tyrosinase locus and flanking upstream regions and improve the probability of isolating the desired non-repetitive sequences.
6. Hypersensitivity Mapping of the Human Tyrosinase Locus

6.1. Results

Four melanogenic cell-specific DHS were identified within the human tyrosinase locus of cultured tyrosinase-expressing SK-Mel-28 human melanoma cells: three upstream of the first exon and one downstream of the fifth exon. Several attempts were made to isolate the genomic region containing sequences representing the -10.5 kb DHS, a possible homologue of the mouse tyrosinase distal upstream enhancer/MAR. These attempts included screening two human genomic DNA bacteriophage libraries, synthesizing and screening an SK-Mel-28 sub-genomic DNA library, and amplifying the region by inverse-PCR.

6.1.1. Tyrosinase Expression in SK-Mel-28 Human Melanoma Cells

There are architectural changes within the chromatin of a gene locus that allow transcriptional regulatory proteins access to their binding sites. Such sites are seen only in those cells that are poised to express the gene in question, are actively doing so, or have a history of expressing that gene. As the purpose of this aspect of the project was to map the human tyrosinase locus for DHS, it was necessary to determine that the SK-Mel-28 human melanoma cell line expressed tyrosinase. Using a human tyrosinase cDNA probe, Northern blot analysis was performed on total RNA isolated from SK-Mel-28 human melanoma cells, U937 human histiocytic lymphoma cells, and SK-N-SH human neuroblastoma cells. The strong positive signal at approximately 1.9 kb on the autoradiogram (Figure 4) corresponds to the full-length human tyrosinase mRNA transcript (Bouchard et al., 1988) and shows that SK-Mel-28 cells express tyrosinase mRNA. The bands below 1.9 kb may represent alternatively spliced tyrosinase transcripts as seen with mouse tyrosinase expression (Ruppert
Figure 4. *Tyrosinase* Expression by SK-Mel-28 Human Melanoma Cells.

**A.** Total cellular RNA from SK-Mel-28 human melanoma cells, U937 human histiocytic lymphoma cells, and SK-N-SH human neuroblastoma cells probed with labelled human *tyrosinase* cDNA isolated from BBTY-1 (Bouchard et al., 1988). Strong hybridization signal at 1.9 kb in SK-Mel-28 lane represents primary *tyrosinase* transcript. No signals are visible in the U937 or SK-N-SH lanes indicating that these cell types do not express *tyrosinase* at detectable levels.

**B.** Agarose gel stained with ethidium bromide prior to Northern blotting showing equivalent amounts of total cellular RNA (10 μg) in each lane.
et al., 1988; Terao et al., 1989; Porter and Mintz, 1991) while the bands larger than 1.9 kb may represent larger transcripts or secondary tyrosinase mRNA structures due to incomplete denaturation. No tyrosinase mRNA was detected in U937 cells or SK-N-SH human neuroblastoma cells indicating that these two cell types do not express tyrosinase at a detectable level. The U937 cell line served as a negative control for the DNase I hypersensitivity mapping.

6.1.2. Restriction Mapping of the Ahtyr34 Bacteriophage Clone

In order to map the far upstream regions of the human tyrosinase locus for DHS, it was necessary to isolate DNA probes representing upstream sequences. The recombinant bacteriophage clone, Ahtyr34, containing upstream human tyrosinase DNA (Giebel et al., 1991) was obtained from Dr. Richard Spritz (Departments of Medical Genetics and Pediatrics, University of Wisconsin). Purified Ahtyr34 was digested with EcoRI and the digests were separated on a 1% agarose gel. The resulting EcoRI restriction fragments were approximately 19.6 kb, 11.5 kb, 5.0 kb, 4.5 kb, 3.5 kb, 2.3 kb, and 1.5 kb (see Figure 5). The 19.6 kb and 11.5 kb fragments represent the long and short arms, respectively, of the Charon 4A cloning vector. The collective length of the remaining fragments was 16.8 kb which was close to the expected length for Ahtyr34 of 16.4 kb as described in Giebel et al. (1991). Two of the obtained EcoRI restriction fragments were expected: the 2.3 kb fragment (htyr2.5E) harbouring exon 1 and immediate 5' and intronic flanking DNA (see Kikuchi et al., 1989) and the 1.5 kb fragment (htyr1.5E) which juxtaposes the 5' end of the 2.3 kb restriction fragment and contains an essential enhancer element required for transcriptional control of human tyrosinase expression (see Ponnazhagan et al., 1994). A Southern blot was
**Figure 5. Restriction Mapping of \( \lambda \text{htyr34} \) Bacteriophage Clone.**

A. *EcoRI* restriction map of \( \lambda \text{htyr34} \) insert in Charon 4A bacteriophage vector showing relative orientation of restriction fragments with detailed enlargement of exon 1 and flanking 5' regions. B, *BglII*; E, *EcoRI*; N, *NsI*I; Nc, *NcoI*; P, *PacI*; S, *ScaI*; X, *XbaI*. Shaded rectangles represent probes generated for mapping and subsequent DNase I hypersensitivity assays of upstream regions of the human tyrosinase gene locus: probe a, 732 bp *RsaI* fragment from human tyrosinase cDNA; probe b, 1.5 kb *EcoRI* fragment (*htyr1.5E*); probe c, 182 bp of PCR-amplified DNA (*htyr0.18US*) upstream of *PacI* restriction site; and probe d, 0.7 kb *EcoRI*/*XbaI* fragment (*htyr0.7EX*) represents the 5' end of *htyr4.5E* and is the same as probe *d* in Figure 6. Solid arrows represent the areas sequenced and the empty arrow heads represent the primers used for PCR amplification.

B. Autoradiogram of Southern blot of *PacI*/*EcoRI* digested \( \lambda \text{htyr34} \) hybridized with probe *c*. The open wedge at the top represents the increasing time of *EcoRI* digestion with time zero represented by gray arrowhead. Molecular weights in kilobases are documented on the left side of the autoradiogram.

C. Autoradiogram of Southern blot of *PacI*/*EcoRI* digested \( \lambda \text{htyr34} \) hybridized with probe *b*. 

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prepared and hybridized with DNA probe a representing the 732 bp RsaI restriction fragment from exon 1 of human tyrosinase cDNA. Probe a hybridized to the 2.3 kb fragment as expected (data not shown), confirming that \( \lambda \text{htyr}34 \) indeed contained the first exon of the human tyrosinase gene. The 5.0 kb, 4.5 kb and 1.5 kb EcoRI fragments were individually subcloned to allow for more detailed restriction mapping of the upstream regions of \( \lambda \text{htyr}34 \).

To determine the relative position of each of the EcoRI restriction fragments within \( \lambda \text{htyr}34 \), the intact clone was first digested with PacI rendering a 15 kb and a 32 kb fragment (see Figure 5). The PacI- digested \( \lambda \text{htyr}34 \) was then partially digested with EcoRI. The restriction digest products were separated by electrophoresis in a 1% agarose gel and two successive Southern blots were prepared from that one gel. Each Southern blot was probed with one of two labelled DNA probes: probe b (htyr1.5E), immediately 3' of the PacI site, or probe c, a 182 bp PCR-amplified fragment (htyr0.18US) representing the 5' side of the PacI site. The Southern blot hybridized with probe c demonstrated 2 bands: one 15 kb and one 4.5 kb representing the short arm of Charon 4A with attached htyr4.5E and htyr4.5E alone, respectively. Probe b hybridized to 5 bands: one over 23 kb and one each at 12.3 kb, 7.3 kb, 3.8 kb, and 1.5 kb. From the Southern results it appeared that the htyr4.5E fragment represents the 5' end of \( \lambda \text{htyr}34 \) and hence, the upstream sequences of the human tyrosinase locus.

To confirm that the most 5' region of \( \lambda \text{htyr}34 \) is htyr4.5E, both ends of the htyr4.5E fragment were sequenced. The 3' end of htyr4.5E showed homology with the previously published sequence of the region flanking the 5' end of htyr1.5E (Ponnazhagan et al., 1994). Restriction mapping of htyr4.5E demonstrated a 5' 0.7 kb EcoRI/XbaI fragment (htyr0.7EX - probe d) which was subcloned and sequenced to confirm its position at the 5' end of
htyr4.5E. Probe d was used to probe a Southern blot of BgII-digested λhtyr34 (data not shown) and hybridized to a 3.5 kb restriction fragment. The distance between the BgII site 1.9 kb in from the 5' end of htyr4.5E and the first BgII site 1.6 kb away from the EcoRI cloning site on the small arm of the Charon 4A vector is 3.5 kb.

6.1.3. DNase I Hypersensitivity Mapping of the Human Tyrosinase Gene Locus

DNase I-treated SK-Mel-28 human melanoma genomic DNA was analysed for the presence of DHS in the human tyrosinase gene locus. Approximately 26 kb of DNA upstream from the first exon and 23 kb of DNA downstream of the fifth exon were analysed and resulted in the detection of four hypersensitive sites. Of the three upstream DHS, one was found to correspond to the promoter region and another to an essential cis-regulatory element. A DHS present 10.5 kb upstream of the transcription initiation site coincided with no known human tyrosinase regulatory element but may indicate the location of the human homolog of the -15 kb mouse tyrosinase enhancer/MAR. One DHS was detected 15 kb downstream of exon five that also does not map to any known human tyrosinase cis-regulatory element.

6.1.3.1. The Promoter and -2 kb Enhancer Element Hypersensitive Sites

Nuclei from the SK-Mel-28 cell line were isolated and incubated with increasing concentrations of DNase I after which the genomic DNA was isolated and purified as described in the Materials and Methods. A NdeI digest was performed on an aliquot of each DNase I concentration and upon completion, the DNA fragments were separated by electrophoresis on a 1% agarose gel and then Southern blotted. The membrane was probed
with a sense riboprobe, probe a1, specific for the first 631 bp of human tyrosinase exon 1 (Figure 6A). The autoradiogram demonstrated 3 distinct hybridization bands (Figure 6B). The 11.7 kb restriction fragment represents the native band (no DNase I) limited by a NdeI site both 10.4 kb upstream and 1.3 downstream of the transcription start site. As the DNase I concentration increased this large band disappeared and a smaller 3.3 kb band appeared representing a fragment limited at the 5' end by a DHS. Ultimately, the 3.3 kb band is replaced by a 1.3 kb band which is limited at the 5' end by a different DHS. The DHS limiting the 1.3 kb fragment represents the tyrosinase promoter region adjacent to the transcription start site (Kikuchi et al., 1989) while the DHS limiting the 3.3 kb fragment corresponds to an essential cis-acting enhancer element found 2 kb upstream of the transcriptional start site (Shibata et al., 1992; Ponnazhagan et al., 1994).

6.1.3.2 A Hypersensitive Site 10.5 kb Upstream of Transcription Start Site

The same SK-Mel-28 human melanoma DNase I series was digested to completion with BgIII, Southern blotted, and probed with htyr0.7XE (probe d). Two distinct bands were evident on the autoradiogram (Figure 6C): a 15 kb native band in the first lane (no DNase I) and a 5.5 kb band that appeared as the DNase I concentration increased with a subsequent loss of the 15 kb band. The 5.5 kb band is limited at its 5' end by the presence of a DHS located at approximately 10.5 kb upstream of the transcription start site. This DHS was confirmed by digesting the SK-Mel-28 DNase I series with either ScaI or NcoI and hybridizing with probe d (Figure 6C). The ScaI digest yielded an approximately 23 kb native band and a 4.2 kb band as the DNase I concentration increased while the NcoI digest demonstrated an 11 kb native band and a 4.4 kb band as the DNase I concentration
Figure 6. Upstream DNase I Hypersensitive Sites of the Human Tyrosinase Gene Locus.

A. Three upstream DNase I hypersensitive sites (DHS; solid arrows) are shown correlating with the promoter, the -2 kb tyrosinase distal enhancer element (TDE), and a possible homologue to the mouse upstream enhancer/MAR at -10.5 kb. The lines underneath the map represent the DNA fragments (with molecular weights in kilobases) obtained upon DNase I/Southern analysis of genomic DNA shown in the autoradiograms in (B) and (C). The probes (shaded rectangles) used for the hypersensitive assays were: probe a1, 631 bp human tyrosinase exon 1 T7/Sty I ribprobe; probe d, htyr0.7EX representing the 5' end of htyr4.5E (Note: the EcoRI site (in brackets) representing the 5' end of htyr4.5E is a cloning artifact and is not found in the SK-Mel-28 genome). E, EcoRI; N, Ndel; B, BglII; N, Ncol; S, Scal; X, Xbal.

B. Autoradiograms of SK-Mel-28 and U937 genomic DNA digested with DNase I and Ndel and hybridized with probe a1. The promoter and the -2 kb DHS are evident in the SK-Mel-28 genomic DNA and the lack of similar DHS in U937 genomic DNA suggests their melanogenic cell-specificity. The molecular weight (kb) of each DNA fragment is noted on the left side of the autoradiograms and the open wedge at the top represents the increasing DNase I concentration with zero DNase I represented by gray arrowhead.

C. Autoradiograms showing the DHS located 10.5 kb upstream of the transcription start site in DNase I/BglII-digested SK-Mel-28 genomic DNA hybridized with probe d. The lack of a corresponding DHS in the U937 control DNA suggests the DHS is melanocyte-specific. Scal I and NcoI digests of DNase I-digested SK-Mel-28 genomic DNA hybridized with probe d confirm the presence of the -10.5 kb DHS.
increased. No corresponding upstream DHS were detected in U937 cells.

6.1.3.3. A Hypersensitive Site Resides 15 kb Downstream of Exon 5

The SK-Mel-28 genomic DNase I-digested DNA was cut with BgIII, Southern blotted and hybridized with a 0.4 kb BgIII/EcoRI fragment (probe e, Figure 7) representing human tyrosinase exon 5 and its 3' flanking sequences. On the autoradiogram, a native band in the control lane of approximately 23 kb representing an intact BgIII restriction fragment was hybridized but as the DNase I concentration increased, this band was replaced by a 15 kb band representing the BgIII fragment limited on its 3' end by a DHS. No corresponding DHS was found in U937 cells.

6.1.3.4. A Tyrosinase-Related Segment is Found on Chromosome 11p

A segment of DNA that has close homology to exons 4 and 5 plus adjacent non-coding regions can be found on chromosome 11p11.2-cen and is termed a 'tyrosinase-related segment (TRS)' (Barton et al., 1988; Giebel et al., 1991). While this truncated sequence may represent a translocated human tyrosinase pseudogene and is not believed to be transcribed in melanogenic cells, alternatively this sequence may be associated with the expression of an unknown gene on 11p in another cell type (Giebel et al., 1991). Given that the length of the TRS downstream of exon 5 is unknown and that probe e used to detect the DHS downstream of exon 5 would also hybridize to a BgIII restriction fragment associated with the TRS, it had to be determined whether the DHS belongs to the 11q tyrosinase gene or to a possible 11p gene flanking the TRS. To accomplish this task, a search was undertaken for polymorphisms involving restriction sites in regions of tyrosinase and TRS...
Figure 7. Downstream DNase I Hypersensitive Site of the Human Tyrosinase Gene Locus.

A. One DNase I hypersensitive site (DHS; solid arrow) 15 kb downstream of the exon 5 is shown correlating with no known human tyrosinase cis-regulatory element. The lines underneath the map represent the DNA fragments (with molecular weights in kilobases) obtained upon DNase I/Southern analysis of genomic DNA shown in the autoradiograms (B). DNA probe e (shaded rectangle) is a 0.4 kb BglI/EcoRI fragment (htyr0.4BE) representing exon 5 coding and 3' untranslated regions. Bg, BglI.

B. Autoradiograms of SK-Mel-28 and U937 genomic DNA digested with DNase I and BglI and hybridized with probe e. The molecular weight of each fragment is displayed on the left side and the open wedge at the top represents increasing DNase I concentration with zero DNase I represented by gray arrowhead.
sequence that reside downstream of the BglII site in exon 5 but not in the TRS, and vice versa. Digesting the DNase I-treated SK-Mel-28 genomic DNA with both BglII and the enzyme with the polymorphic site, as deduced by examining known tyrosinase and TRS sequences (approximately 500 bp, see Giebel et al., 1991) downstream of the exon 5 BglII site, would hopefully render either an 11q tyrosinase gene restriction fragment too short to contain the downstream DHS and a restriction fragment belonging to the TRS that was long enough to harbour the DHS or vice versa. If, after performing a Southern analysis using probe e on DNA cut at both the BglII site in exon 5 and at the chosen downstream polymorphic restriction site found in 11q, the DHS remained, barring an allelic polymorphism, the DHS would be most likely associated with the TRS on 11p. Alternatively, if the downstream polymorphic restriction site were located in the TRS then the DHS would likely belong to the tyrosinase gene. Several candidate restriction site polymorphisms were identified in the fifth exon and 3' flanking sequences, however, the lengths of the resulting restriction fragments were too small to span the distance between the probe and the DHS and as such, the subsequent analysis (data not shown) did not reveal whether the downstream DHS belonged to the human tyrosinase locus or to the TRS.

6.1.4. Isolation of DNA Representing the -10.5 kb Hypersensitive Region

To determine whether the element represented by the distal upstream DNase I DHS is homologous to the -15 kb enhancer/MAR found in the mouse tyrosinase gene locus and to ascertain its possible role, if any, in regulating human tyrosinase gene expression, several attempts were made to isolate DNA representing this region. These isolation attempts included: 1) screening two different human genomic libraries using htyr0.7EX (probe d); 2)
generating a sub-genomic DNA library from SK-Mel-28 genomic DNA and screening it with probe d; 3) performing inverse PCR on SK-Mel-28 genomic DNA using primers complementary to sequences in the htyr0.7EX to amplify the 5' flanking region containing the sequences representing the -10.5 kb DHS.

6.1.4.1. Human Genomic DNA Library Screening

Both the ATCC and Clonetech human genomic DNA bacteriophage libraries were screened following the protocol outlined in the Materials and Methods using probe d. With each screening procedure, a plate containing Ahtyr34 plaques, to which probe d hybridizes, was included to control for each step of the process. No library clones specifically hybridizing to probe d were detected, however, the Ahtyr34 control membranes were positive indicating that the screening procedure was performed correctly.

6.1.4.2. SK-Mel-28 Sub-Genomic DNA Library Construction

Since the -10.5 kb DHS in the human tyrosinase locus was detected using the SK-Mel-28 cell line it became clear that the region harbouring sequences representing the hypersensitive site could be isolated from SK-Mel-28 genomic DNA. An SK-Mel-28 sub-genomic DNA library was prepared as described in the Materials and Methods. Packaging the ligated genomic DNA inserts yielded approximately 1x10^6 recombinant plaques/µg of vector and upon one round of amplification the resulting titre was 4x10^10 pfu/mL. One million pfu of the amplified SK-Mel-28 sub-genomic DNA library were subsequently screened using probe d. No recombinant clones harbouring the 15 kb BgII fragment containing the -10.5 kb DHS region were isolated.
6.1.4.3. Inverse PCR Amplification of the -10.5 kb Hypersensitive Region

Attempts were made to amplify the region containing the -10.5 kb DHS from the sequences flanking *htyr*0.7EX using oligonucleotide primers representing the 5' and 3' ends of the *htyr*0.7EX, oriented in the directions shown in Figure 5, and an inverse-PCR technique. SK-Mel-28 genomic DNA was cut to completion with *Ncol* rendering a population of restriction fragments, some containing the sequences representing the -10.5 kb DHS (see Figure 6). The restriction fragments were circularized and subsequently linearized by cutting at a *NsiI* site that is found 250 bp from the 5' end of *htyr*0.7EX and between the primers (see Figure 5). No *NsiI* site was detected upstream of *htyr*0.7EX for approximately 20 kb (data not shown). The first set of primers used in this assay (set a - see Materials and Methods) primed the amplification of as little as 1.4 pg of the linear 3.7 kb *htyr*0.7EX/pBS II SK+ (*htyr*0.7EX subcloned into pBlueScript cloning vector) control template with the non-linearized *htyr*0.7EX/pBS II SK+ template amplifying approximately five times less efficiently under the same conditions (data not shown). Other control templates that were used included the circular 4.5 kb non-subcloned *htyr*4.5E fragment and the circular 7.5 kb *htyr*4.5E/pBS II SK+ recombinant DNA molecule. An additional *NsiI* site found in *htyr*4.5E, downstream of *htyr*0.7EX, prevented the use of *NsiI* to linearize either the non-subcloned or subcloned *htyr*4.5E templates by cutting between the primers. No other unique restriction site was found between the primers that also would have allowed for linearization of the control templates. No visible amplification was seen using either of the *htyr*4.5E templates or either linear or circular *Ncol*-digested SK-Mel-28 genomic DNA.

Nested primers (set b) were synthesized to determine whether sub-optimally amplified (*i.e.*, non-visible) DNA products of the PCR amplification using primer set a could be
efficiently amplified using primer set b. This was done to enhance the amplification of any linear NcoI-digested SK-Mel-28 genomic DNA that might have been amplified by primer set a but not efficiently enough to generate a visible product by subsequent gel electrophoresis. Primer set b amplified the linear and circular subcloned htyr0.7EX both alone and as a nested primer set after the initial amplification of the htyr0.7EX with primer set a. No other templates, including linear or circular NcoI-digested SK-Mel-28 genomic DNA, were amplified to visibly detectable levels using the nested primer technique. Repeated attempts at optimizing the PCR assay to amplify templates larger than the 3.7 kb htyr0.7EX/pBS II SK+ proved unsuccessful.

6.2. Discussion

6.2.1. DNase I Hypersensitive Sites

Of the three DHS detected upstream of the first exon, two mapped to regions of known regulatory significance. The DHS closest to the first exon co-localized with the promoter region, namely the first 230 bp upstream of the major transcription initiation site. The promoter hypersensitive site was expected because the SK-Mel-28 cell line actively expresses tyrosinase and promoters of active genes are indicated by DNase I hypersensitive regions (Weintraub and Groudine, 1976; Felsenfeld, 1992). Higher resolution mapping of the human tyrosinase promoter region has shown that a multitude of sequence-specific trans-acting regulatory factors bind within the 230 bp region upstream of the transcription start site. Notable consensus binding sequences in the promoter region, demonstrated as genomic DNase I footprints in melanogenic cells (Bentley et al., 1994), include two E-box motifs, an SP-1 element, and an Oct 1 element, all found within 115 bp of the transcription initiation
site. These help to activate tyrosinase expression and may contribute to DNase I hypersensitivity at the human promoter. The -2 kb DHS identified in the present study corresponds to the tyrosinase distal element (TDE), containing two E-box consensus motifs, believed to bind MITF in vivo (Bentley et al., 1994; Yasumoto et al., 1997) and is required to enhance tyrosinase expression above basal levels.

The other two DHS identified in this investigation are novel as well. However, nothing is known of the functional relevance of the elements marked by the distal upstream and downstream DHS to human tyrosinase expression. To date, the study of human tyrosinase transcription regulation to date has been restricted to transient expression analysis of the promoter and the proximal upstream enhancer region (reviewed in Ferguson and Kidson, 1997). The -10.5 kb DHS is an attractive candidate for being the human homologue of the mouse upstream enhancer/MAR because the -10.5 kb DHS is in a similar upstream location and because there are no other detectable DHS among the 16 kb of chromatin assayed upstream of the -10.5 kb DHS. In the mouse tyrosinase locus, the only DHS within the 20 kb of DNA upstream of the transcription start site other than that representing the promoter belongs to the -15 kb enhancer/MAR (Porter et al., 1991). The melanocyte-specific appearance of this DHS certainly suggests that whatever regulatory properties this element may exhibit would be limited to melanocytes. However, structural and functional characterization of this element can only begin once it has been isolated.

The DHS located 15 kb downstream of the human tyrosinase fifth exon maps to no known tyrosinase cis-regulatory element. Indeed, it cannot be stated unequivocally that this DHS maps to the tyrosinase locus as the scenario is complicated by the existence of a TRS that contains exons 4 and 5 and adjacent non-coding sequences. Because of close sequence
homology between the TRS and the tyrosinase gene, probe e used to map the downstream
DHS may be able to hybridize restriction fragments from both. How far past exon 5 the
homology exists between tyrosinase and the TRS is unknown, but it appears that the BgII site
23 kb downstream of exon 5 is shared as a second BgII fragment which also hybridized with
probe e, other than that of 23 kb, was not seen. This presents a conundrum: does the DHS
belong to an unidentified gene flanking the TRS on chromosome 11p or to the tyrosinase
gene? The apparent melanocyte-specificity of the DHS suggests that it belongs to the
 tyrosinase gene and not to the TRS because it would seem unlikely that a gene flanking the
TRS would also be melanocyte-specific and transcriptionally active, or poised to be, at the
same time as tyrosinase. However, multiple unidentified bands are present on the
autoradiogram of the Northern blot analysis for tyrosinase expression by SK-Mel-28 cells
(Figure 4). Since the human tyrosinase probe would also hybridize to the TRS it is possible
that one of these bands represents mRNA from a gene on 11p if the TRS is part of the
transcript. Although Giebel et al. (1991) did not detect any transcripts containing the TRS in
mRNA from FME pigmented human melanoma cells, this does not eliminate the possibility
of expression of such a gene in different cells.

The downstream regions of the mouse tyrosinase locus were examined for the presence
of a similarly positioned DHS. However, it could only be determined with certainty that
DHS were not detectable within 6.5 kb of exon 5 in S91 mouse melanoma genomic DNA
(data not shown). Functional studies have shown that control elements required for
melanocyte-specific, developmentally correct expression of mouse tyrosinase by melanocytes
of both lineages appear to be located upstream and inclusive of the fifth exon. A YAC
containing the mouse tyrosinase gene with 3 kb of 3' and 155 kb of 5' flanking DNA, when
integrated into the genome of albino mice, imparted wild-type levels of coat and eye pigmentations regardless of the position of transgene integration (Schedl et al., 1993; Montoliu et al., 1996). This suggests that all cis-acting elements required for faithful developmental regulation of tyrosinase expression, at least by neural crest-derived melanocytes, were present in the transgene. In the YAC-tyrosinase transgene studies, pigmentation of the eye was assessed on the basis of macroscopic examination only. Whether eye pigmentation was a result of melanogenesis, hence tyrosinase expression, in the neural crest-derived melanocytes of the choroid, the optic cup (neural tube)-derived melanocytes of the RPE, or both, was not addressed. When investigating the -15 kb mouse enhancer/MAR, Porter and Meyer (1994) microscopically observed differences of transgene expression by melanocytes of the choroid and melanocytes of the RPE. They showed that there are different elements that control tyrosinase expression by the two lineages of melanocytes. How the control of tyrosinase expression by RPE differs from that by neural crest-derived melanocytes is just beginning to be determined. Given that they appear to be regulated by distinct elements, the existence of a DHS representing an additional mouse tyrosinase regulatory element downstream of the fifth exon cannot be ruled out.

6.2.2. Isolation of the Region Harbouring the -10.5 kb DNase I Hypersensitive Site

Isolating the -10.5 kb region that harbours the DHS of SK-Mel-28 human melanoma cells is necessary to determine whether this region is both structurally and functionally homologous to the upstream enhancer/MAR found in the mouse tyrosinase gene locus. The presence of a DHS correlates with a cis-active region of functional significance (Gross and Garrard, 1988) and as it appears melanocyte-specific, it is likely that this DHS helps to
regulate human tyrosinase expression in some capacity regardless of whether it is homologous with the mouse enhancer/MAR. Initial attempts at isolating the upstream region containing the DHS sequences were made by screening pre-made genomic DNA libraries. While this region appeared to be hypersensitive only in melanogenic cells, the corresponding sequences should nevertheless be present in all human cell genomes.

A total of $1 \times 10^6$ pfu from the ATCC human genomic DNA library were screened; more than three times the $3 \times 10^5$ clones that are required to provide complete coverage of the genome. That no positive clones were obtained was at first disconcerting as a sufficient number of pfu was screened to detect at least one positive clone from this genomic library. However, the quality and reliability of the library was questioned for two reasons. First, the library was amplified twice: once by the originator (Rotwein, 1986 a,b) and once by ATCC. If the clone containing the hypersensitive region were poorly represented in the original library then there is a possibility that upon two rounds of amplification, the more abundant clones would become much better represented and the clone of interest containing the sequences representing the -10.5 kb DHS could have been lost. Secondly, the titre of the library was determined to be between $1.6 \times 10^8$ and $2.0 \times 10^8$ pfu/mL; 32 to 40% of the $5.0 \times 10^8$ pfu/mL documented titre. A decrease of a DNA library's titre over time is inevitable, however, this significant decrease may have rendered the region containing the DNA sequence corresponding to the -10.5 kb melanocyte-specific DHS absent from this library, especially if it had been poorly represented in the unamplified library. On this basis, a different, possibly more carefully prepared and maintained genomic DNA library was screened.

The Clonetech genomic DNA library had an experimentally determined titre of $4.6 \times 10^{10}$ pfu/mL.
pfu/mL, consistent with the manufacturer's documented titre of \( \geq 10^8 \) pfu/mL. The \( 2.4 \times 10^6 \) bacteriophage screened was a sufficiently large number to ensure coverage of the entire genomic several times over. Since no positive clones were isolated despite the inclusion of a \( \lambda htyr34 \) bacteriophage positive control plate with each screen of both libraries that indicated each step of the screening process was performed correctly, it is concluded that the region of genomic DNA corresponding to the -10.5 kb DHS is poorly represented in these two libraries. Both genomic libraries were prepared by partial digestion of the genomic DNA with either \( MboI \) (ATCC) or \( Sau3AI \) (Clonetech). These enzymes are isoschizomers of each other in that they both recognize the GATC restriction site. It is possible that there is an over-abundance of this recognition site in the regions flanking the -10.5 kb DHS making this region very sensitive to cutting with \( MboI \) or \( Sau3AI \) rendering short restriction fragments that upon ligating with the vector arms yield recombinant molecules that are too short to be packaged into the bacteriophage heads. Consequently, this region would be under-represented in the libraries.

The SK-Mel-28 sub-genomic DNA library construction and inverse-PCR amplification of the SK-Mel-28 genomic DNA were two methods that offered considerable promise for isolating the region containing the sequences representing the -10.5 kb DHS. Successful construction of a sub-genomic DNA library was dependent on selecting the correct size range of \( BglII \) restriction fragments containing the region of interest and controlling the preparatory steps leading to the ligation of the inserts to the bacteriophage vector arms. The \( htyr0.7EX \) probe results of Southern-blotted \( BglII \)-digested SK-Mel-28 DNA suggest that the proper size range of fragments was chosen for the library and the controls, as described in the Materials and Methods appeared to work satisfactorily (data not shown). As the vector arms were
designed to ligate only with the partially filled-in BgII genomic DNA insert fragments, any plaques showing up upon performing a titre and screening the library should have represented recombinants, hence, the background would have been minimal. The actual yield before amplification was 20% of the expected 5x10⁶ recombinant plaques/µg of vector, a poor subcloning efficiency that no doubt had some bearing on the lack of success in isolating a positive clone from the sub-genomic DNA library.

Success with the inverse-PCR suffered most from an inability to optimize the protocol to amplify large molecular weight DNA fragments. Optimization was difficult to achieve primarily because of the lack of proper DNA control templates. The supercoiled conformation that a circular DNA molecule adopts in vivo retards the processivity of DNA polymerase: the efficiency of DNA polymerization is greatly increased when using a linear template (Ausubel, et al., 1994). This is an important consideration especially when long DNA templates are used. Given that the NcoI restriction fragments that contain the -10.5 kb DHS are 11 kb, linearizing the circular genomic DNA templates by cutting between the oligonucleotide primers was imperative in providing the greatest opportunity for successful amplification of this fragment. Unfortunately, the only linear control that was available was the 3.7 kb subcloned htyr0.7EX. The other two controls that contained the oligonucleotide primer sequences, the 4.5 kb htyr4.5E and the 7.5 kb subcloned htyr4.5E, could not be linearized with by any restriction enzyme that cut only between the primers. Successful amplification with both sets of primers was achieved using the recombinant htyr0.7EX/pBS II SK+, with the linear template amplifying far more efficiently than the circular template under the same conditions. The 4.5 kb and 7.5 kb circular templates did not amplify, despite altering many different parameters of the amplification process. As the TaqPlus polymerase
was designed to amplify templates as long as 35 kb, it is likely that the circular nature of the controls was the limiting factor. Without linear control templates of increasing lengths up to 11 kb it is very difficult to implement an optimization strategy and to ultimately determine the ideal conditions for amplifying an 11 kb linear DNA template.

7. Developmental Activation of the Human Tyrosinase Gene

7.1. Results

The upstream regions of the human tyrosinase gene locus of cultured SK-N-SH human neuroblastoma cells and of a cultured subclone of this cell line, SK-N-SHF, were assayed for DHS. Northern blot analysis was performed on cells from both these lines to determine whether tyrosinase was expressed so that a correlation between the transcriptional status of tyrosinase and chromatin structural changes of the tyrosinase locus could be established.

7.1.1. Differentiation and Morphology of SK-N-SH Human Neuroblastoma Cells

The SK-N-SH cell line, established by Biedler et al. (1973) from a bone metastasis, was grown to confluency using the media and conditions outlined in Cell Lines and Culture Methods and was observed to comprise two visibly distinct cell types. The predominantly neuroblastic-like cells with small, round bodies and multiple neurite processes grew in poorly adhering focal aggregates along with interspersed flatter, polygonal-shaped, epithelial-like cells that appeared to adhere more strongly to the culture dish. The cells were grown for 9 days total and were split 1:3 once during that time.

Both the SK-N-SH and SK-N-SHF cell lines have been shown to differentiate from poorly-adherent, neuroblastic-like cells into large, strongly-adherent flat epithelial-like cells.
when cultured in the presence of atRA (Sidell et al., 1983; Slack et al., 1992). With some of the flat cells, this morphological change is concomitant with biochemical changes that are consistent with a melanocytic phenotype (Ross et al., 1983; Ross and Beidler, 1983; Tsokos et al., 1987; Maeda et al., 1990; Slack et al., 1992). SK-N-SHF cells were grown to confluency in the presence or absence of 3X10⁻⁶ M atRA. After 6 days of culture, all cells cultured with atRA had transformed from a neuronal morphology to that of a flat, epithelial-like cell and at day 8, the cells did not appear to undergo any further morphological changes. The cells were grown in the presence of atRA for 6 days - 9 days total culture - and at that time, pigmentation of the transformed cells was not evident. However, refractile bodies were evident by light microscopy, possibly representing pre-melanosomes, that were not present among the non-transformed cells. As expected, the SK-N-SHF cells treated with atRA grew much more slowly than the SK-N-SH cells (see Sidell et al., 1986) and did not have to be split during the 9 day culture. Control SK-N-SHF cells were cultured for 9 days in the presence of the ethanol vehicle alone and had to be split 1:3 once during that time as they grew more quickly than the atRA-treated SK-N-SHF cells. The control cells did not appear to differentiate, maintaining their neuronal morphology.

### 7.1.2 DNase I Hypersensitivity Mapping

Nuclei from SK-N-SH, SK-N-SHF, and SK-N-SHF/atRA cells were DNase I-digested and harvested as previously described. To assay the promoter and upstream regions of the tyrosinase locus for DHS, the DNase I-treated genomic DNA from each cell-type was restriction digested, Southern blotted, and probed in the same manner as the hypersensitivity assays performed on SK-Mel-28 human melanoma genomic DNA. The autoradiogram
results are shown in Figure 8. In each cell type, the absence of DHS at the *tyrosinase* promoter and at the -2 kb enhancer region can be inferred by the lack of 1.3 kb and 3.3 kb fragments respectively as the concentration of DNase I increases (Figure 8A). The faint band at 3.9 kb on the SK-N-SHF/atRA/Ndel autoradiogram does not denote a DHS 2.6 kb upstream of the transcription start site as the band is present in the first lane (no DNase I) and is not present in lane 4 where the 11.7 kb native *Ndel* restriction fragment has almost disappeared. This band possibly represents a recombination event in a subpopulation of *atRA*-treated SK-N-SHF cells. Figure 8B shows that no DHS is present at -10.5 kb. The signal above 15 kb may also represent a mutation or recombination event in a subpopulation of cells.

### 7.1.3. Northern Blot Analysis of Tyrosinase Expression

Total cellular RNA was isolated from the various cell lines, blotted and probed with human *tyrosinase* cDNA to determine the expression of *tyrosinase* (Figure 8C). A strong, positive signal in lane a1, at approximately 1.9 kb, represents the main *tyrosinase* transcript of SK-Mel-28 cells with the other signals likely representing alternatively spliced transcripts or secondary structures of the target mRNA. In lane 4, 20 μg of total SK-N-SH RNA was electrophoresed and probed rather than 10 μg. This was to compensate for the relatively small numbers of flat cells, the ones, if any, that would express *tyrosinase*. No *tyrosinase* mRNA was detected in any of the SK-N-SH or SK-N-SHF cells, *atRA*-treated or otherwise.
Figure 8. DNase I Hypersensitivity and Tyrosinase Expression Analyses of SK-N-SH Human Neuroblastoma Cells.

A. Autoradiograms of DNase I/\textit{Nde I}-digested SK-N-SH human neuroblastoma genomic DNA and SK-N-SHF human neuroblastoma genomic DNA [untreated (center) and treated (right) with \textit{all trans}-retinoic acid (\(a/\text{RA}\))] probed with \textit{probe a1} specific for human \textit{tyrosinase} exon 1 (see Figure 6). The open wedge at the top of the autoradiograms represents increasing DNase I concentration with zero DNase I shown as a gray arrowhead. No DHS are evident that represent the \textit{tyrosinase} promoter and -2 kb TDE. The black arrow on the left side of SK-N-SHF/\textit{arRA/Nde I} autoradiogram denotes a band at 3.9 kb that does not represent a DHS (see section 7.1.2.).

B. Autoradiogram of DNasel/\textit{Bgl II}-digested SK-N-SHF genomic DNA (\textit{a/RA}-treated) hybridized with \textit{probe d} (see Figure 6). No DHS are revealed at -10.5 kb of the human \textit{tyrosinase} locus. The black arrow on the left side of the autoradiogram above the 15 kb band may represent a mutation or recombination event in a subpopulation of cells (see section 7.1.2.).

C. a) Total cellular RNA from the various cell types cultured in the differentiation study probed with human \textit{tyrosinase} cDNA. A strong signal at 1.9 kb in the SK-Mel-28 lane represents the primary \textit{tyrosinase} transcript. No signals are visible in any of the other lanes indicating that \textit{tyrosinase} is not expressed at detectable levels. 1) SK-Mel-28 (positive control), 2) U937 (negative control), 3) SK-N-SH (10 \(\mu\)g), 4) SK-N-SH (20 \(\mu\)g), 5) SK-N-SHF, 6) SK-N-SHF (\textit{a/RA}-treated), 7) SK-N-SHF (ethanol control). b) Agarose gel of electrophoresed total cellular RNA stained with ethidium bromide prior to Northern blotting showing equivalent amounts of total RNA (10 \(\mu\)g) in each lane (except lane 4 which has 20 \(\mu\)g).
7.2. Discussion

SK-N-SHF cells, when cultured in the presence of atRA, transformed from a neuroblastic morphology to a epithelial-like morphology as expected but when assayed for both upstream DHS and concomitant tyrosinase mRNA expression, no DHS or tyrosinase mRNA was detected. No DHS might be expected for the SK-N-SH cells or the SK-N-SHF cells not treated with atRA as tyrosinase activities and melanocyte-associated antigen immunostaining signals are very low, if not detectable, in these cells (Maeda et al., 1990; Slack et al., 1992). However, given that tyrosinase activity is markedly increased in atRA-treated SK-N-SHF cells (Slack et al., 1992) and that mature melanosomes are present in the cytoplasm as well (Tsokos et al., 1987), reorganization of the tyrosinase locus would have had to occur to enable the transcription of tyrosinase and subsequent melanogenesis to occur. This structural alteration should be demonstrable as DNase I hypersensitivity at the tyrosinase promoter and possibly the -2 kb and -10.5 kb enhancer elements. It is possible that some cells had completely differentiated along the melanocytic pathway, as shown by the change of morphology, but were harvested before the structural alteration of the tyrosinase locus had occurred in a sufficient number of cells for the DHS to be detectable by the methods used. Hence, the number of tyrosinase transcripts would likely be below detectable limits on Northern blot analysis as well. Alternatively, all of the cells could have differentiated to a very early melanocytic stage but one not yet detectable by chromatin structural changes or tyrosinase mRNA synthesis.

The transformation of the SK-N-SHF cells to the flat-cell morphology seemed to be complete at day seven of culture and given that it coincided with the appearance, only in the flat cells, of refractile cellular inclusions that were thought to be pre-melanosomes, the
decision was made to harvest the cells for the hypersensitivity and mRNA assays. In
previous differentiation studies that showed tyrosinase activity and melanogenesis by atRA-
transformed SK-N-SHF cells, the cells were cultured for a period of 12 to 14 days total (9 to
11 days in the presence of atRA) (see Sidell et al., 1986 & Tsokos et al., 1987). Thus, it is
possible that the SK-N-SHF cells in this study were not exposed to atRA for a sufficient
length of time to induce detectable structural changes in the tyrosinase locus.

Another possibility that no DHS or tyrosinase mRNA were detected is that the majority
of flat cells that were present were not melanocytic but were Schwann cells. In human
neuroblastoma, Schwannian and melanocytic differentiation often occurs in conjunction with
each other (Tsokos et al., 1987) and the degree to which one cell type predominates appears
to be influenced by culture conditions such as plating density (Tsokos et al., 1985). In the
absence of an easily identifiable melanocytic phenotype, such as pigmentation, one could
determine whether the observed cells were melanocytic or Schwannian by assessing the
extracellular matrix proteins elaborated by the cells (Tsokos et al., 1987), by determining
whether the Schwann cell-associated enzyme, CNP (2',3'-cyclic nucleotide-3-
phosphohydrolase) is actively expressed (Tsokos et al., 1987), or by immunostaining for
melanocyte lineage-specific antigens (Maeda et al., 1990; Slack et al., 1992).

There is strong evidence in the literature to suggest that the SK-N-SH human
neuroblastoma cell line might be a viable model with which to study the activation of the
tyrosinase gene locus. However, based on the results presented here, the only conclusion
that can be made which supports that notion is that the SK-N-SHF cells differentiated to a
flat-cell phenotype in the presence of atRA which is consistent with the transformation of the
neural crest-derived cell line from neuronal-type cells to melanocytic/Schwannian cells
observed in many studies.

8. The Human Tyrosinase Coding Region Exhibits Repressor Activity

8.1 Results

Recombinant *luciferase* reporter gene constructs, driven either by the mouse *tyrosinase* promoter or a heterologous SV40 early promoter and containing all or parts of the human *tyrosinase* cDNA (Figure 9A), were generated and transiently transfected, in triplicate, into cultured S91 mouse melanoma cells along with equimolar amounts of the *pSV-β-galactosidase* reporter plasmid to control for transfection efficiency differences between the constructs (see Materials and Methods). The luciferase/β-galactosidase activity values (counts per minute, CPM) for each construct were plotted relative to the luciferase/β-galactosidase activity values for the respective non-recombinant, promoter-only *luciferase* reporter gene plasmids (Figure 9B). A decrease of luciferase activity is clearly seen when the reporter construct contains all or parts of the human *tyrosinase* cDNA.

The most dramatic drop of luciferase activity, a 90% decrease, is evident in cells transfected with the SV40 early promoter-driven construct containing the first 59 bp of human *tyrosinase* exon 1 (*pGL23'x1b*). Attempts at constructing *pluc3'x1b* proved unsuccessful so it is unknown whether the repression of *luciferase* by these sequences is limited to the construct containing the heterologous promoter. However, it appears that the other six constructs, *pGL2/pluc3'htyr*, *pGL2/pluc3'x1*, and *pGL2/pluc3'x25*, are able to repress *luciferase* transcription, to varying degrees, independently of the promoter. Luciferase activity was decreased by 32% or 34% in S91 cells transfected with *pGL23'x25* or *pluc3'x25*, respectively, suggesting that repression may also be mediated through one or
Figure 9. Luciferase Reporter Gene Constructs and Graphical Analysis of Luciferase Repression by Human Tyrosinase cDNA.

A. Schematic illustration showing the constructs used for the transient transfection analyses of the repressive effects of human tyrosinase cDNA. The pGL2 constructs contained the SV40 early promoter while the pluc constructs contained the mouse tyrosinase promoter. The human tyrosinase cDNA sequences were subcloned, in a 3' to 5' direction, into the BamHI and SalI sites downstream of the luciferase coding sequences. pGL2/pluc3'h tyr, entire tyrosinase cDNA; pGL23'x1b, 59 bp of exon 1; pGL2/pluc3'xl, exon 1 and half of exon 2; pGL2/pluc3'x25, exons 5, 4, 3, and half of exon 2.

B. Graph showing luciferase activity of cell extracts from cultured S91 mouse melanoma cells transiently transfected with the various luciferase constructs containing all, none, or parts of the human tyrosinase cDNA. The white bars represent the pluc constructs and the stippled bars represent the pGL2 constructs. The error bars represent the SEM.

C. Graph showing luciferase activity of cell extracts from cultured SK-Mel-28 human melanoma cells (black bars), cultured HeLa human cervical epithelioid carcinoma cells (stippled bars), and cultured S91 mouse melanoma cells (white bars) transfected with pGL2pro, pGL23'h tyr, or pGL23'x1b. The error bars represent the SEM.
more elements in addition to the one(s) that may reside in exon 1. It would also appear that
some manner of repressive cooperation or additive effect exists as the entire human
tyrosinase cDNA (pGL2/pluc3’htyr) represses luciferase expression to a greater degree (by
76% and 82%, respectively) than do either pGL/pluc23’x1 alone (by 70% and 64%,
respectively) or pGL2/pluc3’x25 alone.

To determine whether the repressive effect of human tyrosinase cDNA on luciferase
expression is species- and cell type-specific, the pGL2pro, pGL23’htyr, and pGL23’x1b
plasmids were transiently transfected into cultured SK-Mel-28 human melanoma cells and
cultured human cervical epithelioid carcinoma cells in the same manner as with the S91
mouse melanoma cells. A pattern of luciferase repression (Figure 9C) similar to that of the
S91 cells was observed in both of the other cell types suggesting that the repression is neither
cell type-specific nor species-specific.

As the greatest degree of luciferase repression was observed in cells transfected with the
pGL23’x1 construct, the sequence of the first 59 bp of the human tyrosinase cDNA was
analyzed for known repressor elements using MatInspector v2.2 (Quandt et al., 1995).
Potential binding sites for the following proteins were identified: AP4, IK1, IK2, NF1,
NFκB, and Th1/E47.

8.2 Discussion

The repression of luciferase in those cells transfected with human tyrosinase cDNA-
containing reporter gene constructs is interesting as it may explain pigmentation differences
between albino mice harbouring human tyrosinase cDNA transgenes and those transgenic for
mouse tyrosinase cDNA. Lines of transgenic mice (TgPT) generated by Porter and Meyer
(1994), containing human tyrosinase cDNA, 2.5 kb of the mouse tyrosinase promoter and none of the -15 kb enhancer/MAR region, demonstrated very low levels of coat and eye pigmentation. In the two mice that were visibly pigmented, only small amounts of pigment were present and only in the tail, ear, scrotum and eyes while transgenic mice containing the same mouse tyrosinase promoter sequences but fused with the mouse tyrosinase cDNA (Tanaka et al., 1990) or mini-gene (Yokoyama et al., 1990) demonstrated higher levels of pigmentation. This evidence suggests that a negative regulatory element may exist within the human tyrosinase cDNA, a notion which is supported by the transient transfection analyses performed here.

It is unlikely that the observed repressive effects of the luciferase gene by human tyrosinase cDNA is artifactual. The pGL2/pluc3'htyr, pGL2/pluc3'x1, and pGL2/pluc3'x25 constructs were all made from the unadulterated reporter gene plasmids and intact human tyrosinase cDNA so any damage introduced to the luciferase gene or associated cis-regulatory elements as a result of subcloning would be limited to the affected construct and would not be carried over to subsequent constructs. Artifactual luciferase repression effects due to DNA inserted downstream of the luciferase coding sequences have not been reported so it is unlikely that the mere presence of exogenous DNA in the downstream BamHI/Sall sites would cause the observed repression unless the inserted DNA mediates repressor activity.\(^1\)

The results of these expression studies are inadequate to define a likely mechanism to account for the repressor activity observed. However, there are four observations that may

\(^1\) Based on discussions with the technical staff at Promega, the manufacturer of the pGL2-promoter and pGL2-basic luciferase reporter plasmids.
ultimately help to explain, or exclude, possible mechanisms: 1) cooperative or additive repression may exist between the putative repressor element(s) of the human tyrosinase first exon and the putative element(s) of the remaining coding sequences; 2) repression is mediated over a relatively long distance; 3) repression is promoter-independent; 4) repression is cell type- and species-independent. Cooperative repression is seen with other genes such as the yeast silent mating loci, HML, where the silencing activity of the essential (E) and important (I) repressor elements is significantly augmented by the presence of Rap1 (repressor activator protein 1) and/or Abf1 (autonomous replication sequence binding factor 1) binding sites located over 4 kb away from either E or I (Boscheron et al., 1996). The repressor activity on the luciferase gene occurs over a relatively large distance as well: the putative repressive elements in the human tyrosinase cDNA are approximately 3 to 4.8 kb downstream of the SV40 and mouse tyrosinase promoters. This suggests that a looping of DNA may be required to allow interaction between the promoter and silencer elements (Boscheron et al., 1996; Hanna-Rose and Hansen, 1996). However, in the tyrosinase gene's natural environment, the putative silencer element(s) somewhere in exons 2 through 5 would be separated from the promoter and putative repressor element(s) in exon 1 by a considerable distance (up to 50 kb). It is difficult to predict whether the cooperative (or any) repression is physiologically relevant with respect to melanogenesis in the native setting or is a coincidental result of bringing the coding sequences close together in the cDNA (Fallaux et al., 1996).

The apparent promoter-independence of the silencing activity in a non-species- and non-cell-type-specific fashion, could limit the potential mechanisms of repression to those that interfere with the assembly or activity of the basal transcription complex, otherwise known as
active repression. This is in contrast to quenching repressors that suppress gene expression by interfering with promoter activator proteins and which tend to repress transcription only at certain promoters as the activators are frequently promoter-specific (Hanna-Rose and Hansen, 1996). Active repressors are important for genes that are expressed in a cell-specific manner - which tyrosinase is - as they allow a gene to be effectively turned off in those tissues in which the gene is not to be expressed (Johnson, 1995).

Three sets of sequences in the pGL23'x1b human tyrosinase cDNA resemble protein binding sites that have demonstrated transcriptional repression activity in various contexts: a NF1 (nuclear factor 1) site at position 54, a NFκB (nuclear factor κB) site at position 37, and a Th1 (Thing 1) site at position 41. Members of the NF1 family of ubiquitous transcription factors have been shown to bind silencer elements and repress expression of the rat GST-P (glutathione transferase-P) gene, prevent transcriptional activation of the human metallothionein II A promoter (Osada et al., 1997 a,b) and are required to repress β-major-globin gene expression by mouse erythroleukemia (MEL) cells (Macleod and Plumb, 1991). NF1 is thought to mediate repression by directly interacting with the general transcription machinery (Osada et al., 1997b). NFκB normally functions as a transcriptional activator of several promoters. However, activation can be repressed by the binding of RBP-Jκ (recombination signal sequence binding protein-Jκ), present in most cells, to the NFκB binding site (Plaisance et al., 1997). A degenerate E-box motif resembling the binding site for the Th1/E47 bHLH heterodimer was identified at nucleotide 41. Th1 (Thing 1) acts as a transcriptional activator when complexed with E protein (i.e., E47) but the HLH domain of Th1 has been shown to mediate transcriptional repression of CAT in transiently transfected NIH 3T3 fibroblast cells, a repression which can be alleviated somewhat by E protein.
No tyrosinase transcriptional regulatory activity has been previously attributed to any cis-regulatory elements in the human tyrosinase gene coding regions (reviewed in Ferguson and Kidson, 1997). It is not known whether the above binding sites in the first exon and their associated proteins even affect the regulation of tyrosinase expression but given the strong repressive activity observed, these elements are promising candidates for function in that capacity. As it is difficult to assess the significance of the putative NF1, NFκB, Th1/E47 binding sites in the first exon based solely on their presence, performing similar transient transfection expression assays, perhaps by linker scanning mutagenesis of the mouse tyrosinase sequences in pGL23'x1b, might pinpoint one particular element or region that is instrumental in effecting the observed luciferase repression.

9. Summary and Future Directions

The two main hypotheses upon which this research was based were: 1) the DHS/MAR located 15 kb upstream of the transcription initiation site in the mouse tyrosinase gene is the 5' domain boundary of the tyrosinase locus and 2) an upstream LCR-like element, homologous to the -15 kb mouse tyrosinase enhancer/MAR, is present in the upstream regions of the human tyrosinase locus. The results of the general DNase I sensitivity assays of the mouse tyrosinase locus in tyrosinase-positive cells suggest that the -15 kb DHS/MAR represents a point of structural transition with the chromatin immediately upstream of this element displaying a DNase I sensitivity approaching that of heterochromatin. Thus, the DHS/MAR appears to delimit a region of increased nuclease sensitivity and may be the 5'
domain boundary of the mouse tyrosinase locus. However, the evidence for this is not unequivocal. If non-repetitive sequences upstream of the LINE flanking the MAR could be isolated and used to show that the chromatin upstream of the DHS/MAR is as equally sensitive to DNase I as is bulk chromatin then it could be stated with more certainty that the DHS/MAR is the 5' domain boundary of the mouse tyrosinase locus.

The mechanisms underlying the ability of a MAR to insulate against position effects are not well understood, in part because they appear to function dissimilarly in different genes and contexts. MARs defy a simple functional definition. A question that is just beginning to be answered is how does the putative 5' boundary MAR of the mouse tyrosinase locus work? Unlike the A-elements of the chicken lysozyme gene, which do not contribute to locus activation and position-independent expression by transgenic mice but merely protect the lysozyme transgene from being expressed ectopically (Bonifer et al., 1994), tyrosinase transgene activation and position-independent expression requires the -15 kb enhancer and MAR to work cooperatively (Porter and Meyer, 1994). In this context, the transgene needs only to have the MAR at one end for it be expressed independently of position. But when the MAR alone is present, it can insulate a transgene from position effects only when the transgene is flanked at both ends by the MAR (Porter et al., unpublished). Even then, the ability of the MAR to insulate against position effects also appears to depend on the type of melanocyte (Porter et al., unpublished). It would be interesting to see if these insulative capacities can be demonstrated in an enhancer-blocking assay similar to the ones used by Kellum and Schedl (1992) or Cai and Levine (1995). In the former study, a MAR from the intergenic spacer separating the two hsp70 genes in the Drosophila 87A7 heat shock locus was unable to block the yp-1 promoter from activating β-galactosidase expression.
However, this MAR was not a boundary element and has not been shown to have insulative properties.

DNase I hypersensitivity mapping of the human tyrosinase locus revealed four novel DHS, one of which resides 10.5 kb upstream of the transcription initiation site making it a good candidate for the human homologue to the LCR-like enhancer/MAR upstream element in the mouse locus. As the -10.5 kb DHS and the DHS discovered 15 kb downstream of the fifth exon likely represent elements of cis-regulatory significance, isolating these elements and determining how and where they influence transgene expression in albino mice would help establish how they might function to regulate human tyrosinase expression in melanocytes of both lineages. While definitive results were lacking in the tyrosinase gene activation experiments, the SK-N-SH human neuroblastoma cell line still holds promise as a model with which to follow the developmental activation of the human tyrosinase gene of neural crest-derived melanocytes. Developing this model will facilitate the examination of how these elements, represented by the distal upstream and downstream DHS, influence the regulation of tyrosinase expression.

A benefit of understanding how and what structural elements affect higher order gene control is that it can be applied to the promising field of gene therapy. Successful gene replacement or augmentation therapy relies on the ability of a therapeutic transgene to be faithfully expressed once it is taken up by the target cell. However, for a transgene to remain viable for the life of the target cell, it must be stably integrated into the genome. As a result, the potential for position effects is substantial, particularly if a transgene is not provided with a means of protection against the transcriptionally suppressive effects of heterochromatin. In addition, the cis-regulatory elements that are required for the faithful
expression of a therapeutic transgene may activate the undesirable expression of a closely positioned gene, perhaps an oncogene, upon integrating into the genome. Determining what elements are required to not only activate a transgene and to insulate it from such deleterious effects but to safeguard against the spurious activation of other genes as well is paramount if somatic or germ-line gene therapy is to be an effective treatment for genetic disease.
10. References


